DOCTORAL THESIS

Drug targets for urinary and faecal incontinence and anal fissures

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Drug targets for urinary and faecal incontinence and anal fissures

PhD Thesis

By

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ABSTRACT

As life expectancy increases due to better diagnosis and treatments for chronic diseases, the ageing population and the number of people suffering from incontinence will increase. Thus, although non-life threatening, there is a need for better treatments for these conditions.

Correct functioning of the urethra and the anorectum is reliant on the smooth muscle of these tissues. The internal anal sphincter of the anorectum provides most of the resting anal pressure, while the urethral circular smooth muscle provides the resting urethral pressure. Loss of tone in these smooth muscles is associated with faecal incontinence and stress urinary incontinence respectively. Presently, there are no effective pharmacological treatments for stress urinary incontinence or faecal incontinence. Adverse side effects have hampered the development and use of pharmacotherapies for these conditions, mainly due to a poor understanding of urethral and anorectal function.

The aim of this PhD project was to increase our understanding of the pharmacology of the urethra and the anorectum. Specifically, the intent was to elucidate the pharmacology of the smooth muscle of the urethra and the anorectum; to establish the influence of the urothelial lining on urethral contraction; to investigate the mediators and neurotransmitters involved in the regulation of these tissues and to characterise the intracellular signalling pathways involved in contraction and relaxation.

Regional differences, age-related differences and signalling pathways in response to G-protein-coupled receptor activation were investigated directly in the urethra, using an in vitro organ bath technique with isolated porcine tissues (Large white-Landrace), whilst the neurotransmitters of the internal anal sphincter were investigated using electrically field stimulated porcine tissues.

Using these techniques, a role for cellular Ca^{2+}, protein kinase C and Rho kinase in influencing the basal tone and receptor-mediated responses of the urethra was investigated. Experiments investigating urethral α_1-adrenoceptor-mediated responses showed the proximal urethra to be the region with the greatest contractile response, and this was similar in tissues from young (6 months) and older (36 months) pigs. The urothelium/lamina propria of the

urethra had an inhibitory effect on the underlying smooth muscle that did not involve prostaglandins or nitric oxide.

Ca^{2+}-sensitization which is the increase in Ca^{2+}-sensitivity of the contractile apparatus, prostaglandins and Ca^{2+} influx via L-type Ca^{2+} channels all contributed to the basal tone developed by the urethra. Likewise, pharmacological inhibition experiments showed that the potency of the α_1-adrenoceptor agonists was proportional to their ability to induce Ca^{2+} sensitisation. A61603, a specific α_{1A}- adrenoceptor agonist, produced a long-lasting contractile tone, whilst tone was not maintained following stimulation with noradrenaline.

Incubation of tissues with A61603 and phenylephrine caused desensitisation of subsequent responses to noradrenaline. Rho kinase and protein kinase C activity, as well as cytoplasmic Ca^{2+}, contributed to the A61603-induced desensitisation, whilst only Rho kinase contributed to phenylephrine-induced desensitisation. These results suggest Rho kinase involvement in a feedback mechanism to prevent chronic overstimulation of the α_1-adrenoceptor in the urethra. Prior activation of muscarinic receptors before activation of α_1-adrenoceptors had no significant effect on α_1-adrenoceptor-mediated responses in the presence of the urothelium/lamina propria. However, in the absence of the urothelium/lamina propria, prior activation of muscarinic receptors significantly reduced the α_1-adrenoceptor-mediated responses, suggesting the modulation of receptor interactions by the urothelium/lamina propria. This interaction was independent of neuronally released factors or prostaglandins.

Finally, this study showed co-transmission in the internal anal sphincter, with neurogenic contraction mediated by noradrenaline and ATP, whilst neurogenic relaxation of the porcine internal anal sphincter was mediated by the simultaneous release of the gasotransmitters nitric oxide, carbon monoxide and hydrogen sulphide, with relative contributions of nitric oxide > carbon monoxide > hydrogen sulphide. Neurotransmission in the pig internal anal sphincter does not appear to involve acetylcholine. All of these transmitters represent possible targets for drug development, where enhancing sphincter tone may aid the treatment of faecal incontinence or reductions in internal anal sphincter tone may aid healing of anal fissures.

Based on these observations Ca^{2+} sensitization is a significant pathway involved in the mediation of urethral contractile responses and basal tone, as well as modulation of α_{1A}-

adrenoceptor desensitisation. Thus, the Ca^{2+} sensitization pathway stands out as a prospective drug target for the development of new treatments for lower urinary tract symptoms such as stress urinary incontinence.

Further research is necessary to determine the relative contribution of the Ca^{2+} sensitization pathways in incontinence, as there is the possibility of a switch towards a reduced Ca^{2+} sensitization mechanism, which could underlie stress urinary incontinence and faecal incontinence, whilst greater Ca^{2+} sensitization may underlie hypertonia of the internal anal sphincter resulting in an inhibition of healing of anal fissures.

DECLARATION OF ADDENDUM

‘The work presented in this document has not been submitted for a degree or any other purpose at this university or any other institution. To the best of my knowledge, this report has no material already published or written by any other person except where due reference is made’

Signed:

Oladayo Seun Folasire

Date: September, 2016.

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Abbreviations

AC: Adenylate cyclase

AMP: Adenosine monophosphate

AP-2: Adaptor protein complex 2

ATP: Adenosine triphosphate

BK: Bradykinin

BOO: Bladder outlet obstruction

CD117: Cluster of differentiation 117 protein

CO: Carbon monoxide

CPI-17: PKC-potentiated inhibitory protein for protein phosphatase-1 of 17KDa

CREB: Cyclic AMP-response element binding protein

CRELD1: Cysteine Rich With EGF Like Domains 1

EFS: Electrical field stimulation

ET: Endothelin

GMP: Guanosine monophosphate

GPCR: G-protein-coupled receptor

GRK: G-protein-coupled receptor kinase

H_2S: Hydrogen sulphide

IAS: Internal anal sphincter

ICC -Interstitial cells of Cajal

ICC-LCs -Interstitial cells of Cajal -like cells

LP: Lamina propria

mAChR: Muscarinic acetylcholine receptor

MAPK: Mitogen-activated protein kinase

MYPT1: Myosin phosphatase target subunit 1

NO: Nitric oxide

NOS: Nitric oxide synthase

OAB: Overactive bladder

ODQ: 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one

PG: Prostaglandins

PI3K: Phosphoinositide-3-kinase

QOL: Quality of life

RhoA: Ras homolog gene family, member A

ROCK: Rho-associated coiled-coil forming protein serine/threonine kinase (Rho kinase)

SEM: Standard error of mean

SUI: Stress urinary incontinence

TEA: Tetraethylammonium chloride

VIP: Vasoactive intestinal peptide

PUBLICATIONS

Publications

Folasire O, Mills KA, Sellers DJ, Chess-Williams R (2016). Three gaseous neurotransmitters, nitric oxide, carbon monoxide and hydrogen sulphide, are involved in the neurogenic relaxation responses of the porcine internal anal sphincter**.** *Journal of Neurogastroenterology Motility* **22** (1); 141-148.

Abstracts

- **Oladayo S. Folasire**, Donna J. Sellers & Russ Chess-Williams; Desensitisation of urethral smooth muscle: interaction between adrenoceptor and muscarinic receptor-mediated pathways. Abstract presented at ICS 2015 (Biomarkers and Molecular Mechanisms), Montreal, Canada. http://www.ics.org/Abstracts/Publish/241/000470.pdf.
- Donna J. Sellers, **Oladayo S. Folasire** & Lee S; Streptozotocin-induced diabetes increases intravesical ATP release and nerve-evoked contractions in the rat urinary bladder. Abstract presented at ICS 2015 (Biomarkers and Molecular Mechanisms), Montreal, Canada. http://www.ics.org/Abstracts/Publish/241/000152.pdf.
- **Oladayo S. Folasire**, Donna J. Sellers & Russ Chess-Williams; KCl potentiates subsequent responses to noradrenaline of the porcine urethra by enhancing ROCK and Ca^{2+} release. Abstract presented at ASCEPT-MPGPCR 2014 Joint Scientific Meeting. Melbourne Australia. http://www.asceptasm.com/wp-content/uploads/2014/01/ASCEPT-Poster-ProgramAbstracts-FINAL1.pdf.
- Russ Chess-Williams, **Oladayo S Folasire**, Kylie Mills, Donna Sellers. Characterisation of relaxatory transmitters in the porcine internal anal sphincter Abstract presented at ASCEPT-MPGPCR 2013 Joint Scientific Meeting. Sydney Australia, abstract 434, 2013. http://www.asceptasm.com/wp-content/uploads/2013/06/ASCEPT-abstracts-403-493.pdf.
- Donna Sellers, **Oladayo S Folasire**, Russ Chess-Williams. Characterisation of contractile responses to α_1 adrenoceptor agonists in the porcine urethral circular smooth muscle. Abstract presented at ASCEPT-MPGPCR 2013 Joint Scientific

Meeting. Sydney Australia, abstract 435, 2013. http://www.asceptasm.com/wp-content/uploads/2013/06/ASCEPT-abstracts-403-493.pdf.

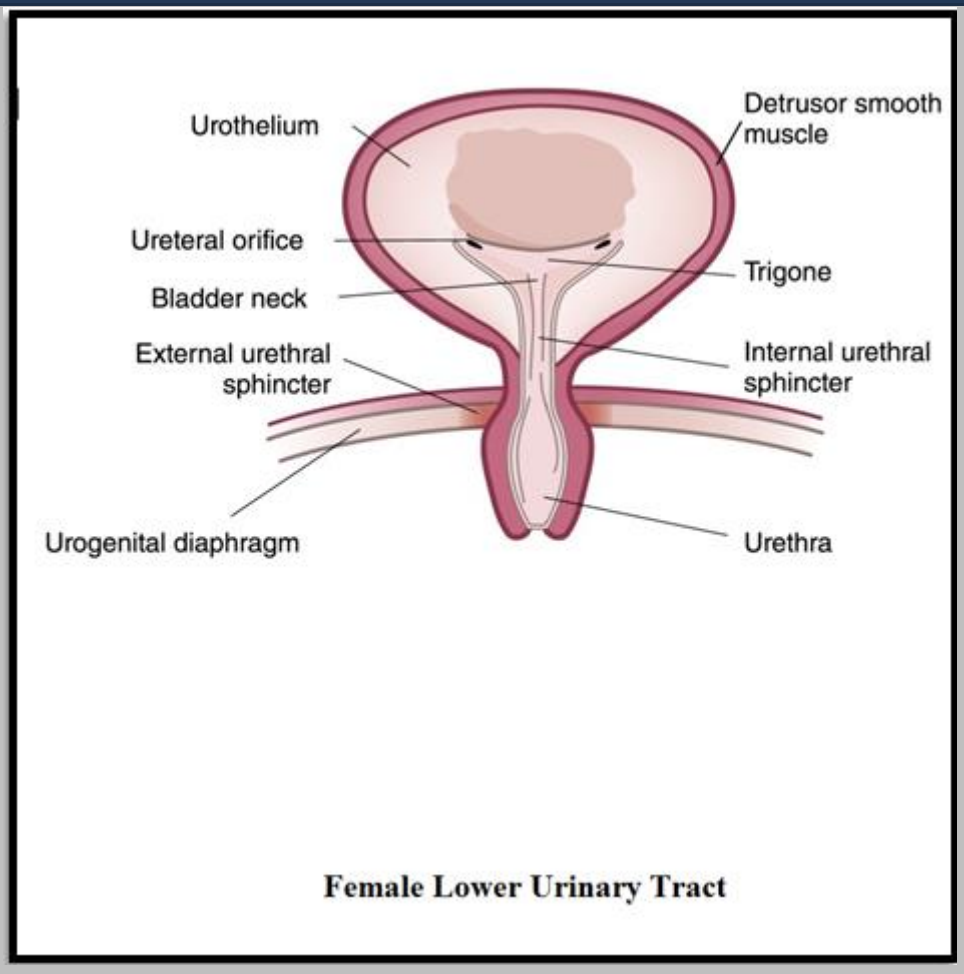
- Russ Chess-Williams, **Oladayo S Folasire**, Kylie Mills. Characterisation of relaxatory transmitters in the porcine internal anal sphincter. Abstract presented at ASCEPT-MPGPCR 2013 Joint Scientific Meeting. Sydney Australia, abstract 435, 2013. http://www.asceptasm.com/wp-content/uploads/2013/06/ASCEPT-abstracts-403-493.pdf.

Presentations

- **Oladayo S. Folasire**, Donna J. Sellers & Russ Chess-Williams; Interaction of adrenergic and muscarinic pathways in the porcine urethra. Presented at 6th National Symposium on Advances in Gastrointestinal and Urogenital Research, 2014.
- **Oladayo S. Folasire**, Donna J. Sellers & Russ Chess-Williams; Characterisation of contractile responses to α_1 adrenoceptor agonists in the porcine urethral circular smooth muscle. Presented at 5th National Symposium on Advances in Gastrointestinal and Urogenital Research, 2013.

CONTENTS

CHAPTER 1



Female Lower Urinary Tract

Modified from Hill, 2015.

1 GENERAL INTRODUCTION

Approximately 1.9 billion individuals worldwide experience lower urinary tract symptoms, and this prevalence is predicted to increase by 18.4% to 2.3 billion by 2018 (Irwin et al., 2011). Worldwide, across Europe, the USA and Asia, the prevalence of urinary incontinence ranges from 21%-65.0% (Ge et al., 2015; Hunskaar et al., 2004; Liu et al., 2014; Markland et al., 2011; Shin et al., 2009). In Australia in 2005 over 2 million people were urinary incontinent, while over 1 million were faecally incontinent (Chiarelli et al., 2005). There is a greater prevalence in women than men, although beyond age 60, the percentage of incontinent males increases almost to that of incontinent females, due to incontinence associated with prostate surgery (Buckley et al., 2010).

Incontinence severely affects quality of life. It is responsible for social isolation, depression, loss of productivity, unemployment, anxiety and also poses an enormous economic burden (Bogner et al., 2002; Chong et al., 2011; Melville et al., 2005; Xu et al., 2012; Yip et al., 2013). The cost of treatment and care of patients with incontinence is significant, with the institutionalisation of patients compounding the cost (Wilson et al., 2001). The annual cost of care for patients with urinary incontinence in the USA was estimated to be approximately $16.3 billion in the community and nursing homes (Wilson et al., 2001). Moreover, Australia spent $710.44 million in 1998 on healthcare for urinary incontinence; approximately $387 per person, with total personal expenditure around $371.97 million (Doran et al., 2001).

1.1 THE LOWER URINARY TRACT

1.1.1 Structure

In males, the lower urinary tract is made up of the urinary bladder, urethra and prostate (Figure 1.1A), while in females the prostate is absent (Figure 1.1B). The bladder is a pyramid shape structure, which lies entirely within the pelvis when empty. Upon filling, the bladder rises into the abdominal cavity (Shah et al., 2014).

There are two parts to the bladder: the body, lying above the ureteral orifices, and the base, consisting of the trigone and bladder neck (De Groat & Yoshimura, 2015).

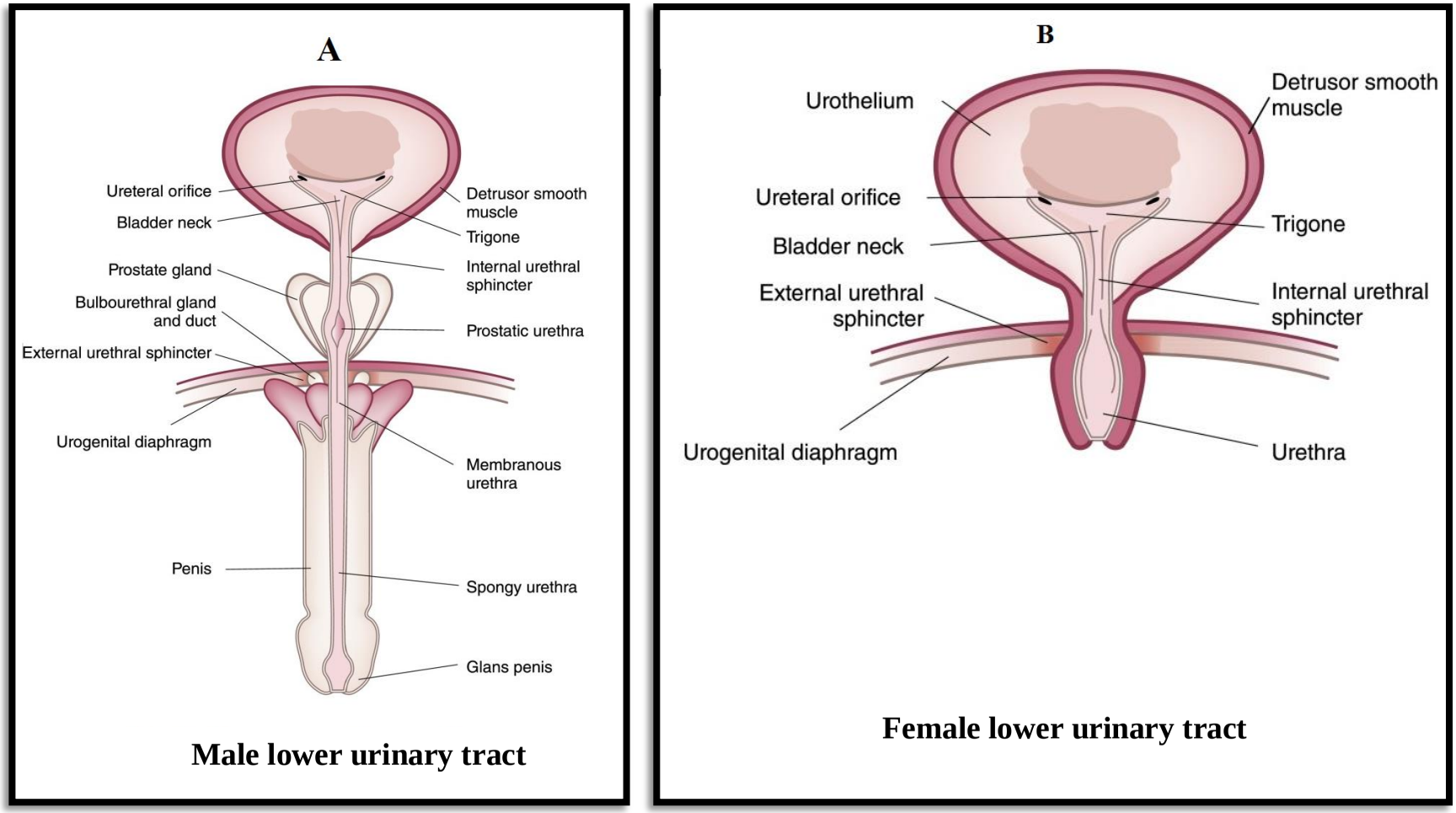


Figure 1.1 The male and female lower urinary tracts. The lower urinary tract consists of the bladder and urethra. The male urethra (A) differs from the female (B) since the male urethra is surrounded by the prostate and is longer. Adapted from (Hill, 2015).

The bladder dome is the greater part of the bladder body. The entry of the ureters marks the triangular area trigone within the base of the bladder (Shah et al., 2014). The distance between the ureteral orifices is usually around 2.5 cm extending up to 5 cm when the bladder is full. The trigone is histologically and embryologically different from the rest of the bladder and contains a rich plexus of neuronal tissue. It is also the least movable part of the bladder and is adherent to the underlying muscle (Mangera et al., 2013).

The bladder wall is composed of the mucosal layer, the muscularis propria and the adventitia (Aydin et al., 2002). The bladder mucosa consists of the urothelium, a basement membrane, and the lamina propria, which contains the vascular system and sensory nerves (Figure 1.2) (Aydin et al., 2002; Wiseman et al., 2002). Bladder wall thickness ranges between 2.0 and 3.4 mm in humans and is greater in men than women (Hakenberg et al., 2000; Kanyilmaz et al., 2013). Moreover, the thickness of the bladder wall depends on the degree of distension by the contained urine (Kanyilmaz et al., 2013).

The bladder neck is the anatomical area of the bladder outlet and the entrance to the urethra. It is formed by several structures, including the detrusor smooth muscle, the vesical sphincter, and adjacent proximal prostatic tissue in males (Li et al., 2015).

The urethra is a smooth muscle tube, attached proximally to the bladder, and surrounded distally by a skeletal muscle ring (the rhabdosphincter), and through which urine is expelled out of the body (Figure 1.1). The urethra begins at the internal meatus of the bladder and extends to the external meatus, and is composed of an inner longitudinal and middle circular smooth muscle layer (Figure 1.2) (Pradidarcheep et al., 2011).

In the male, four segments of the urethra are readily identified. The first is the pre-prostatic portion, or the bladder neck, which consists of a complete circular collar of smooth-muscle cells that extends distally to surround the proximal part of the urethra (De Groat & Yoshimura, 2015; Li et al., 2015). The prostatic urethra (second segment) extends throughout the length of the prostate gland, terminating at its apex. The membranous urethra (third segment) extends from the prostatic apex through the pelvic floor musculature forming the bulbous and penile urethra (fourth segment) at the base of the penis (Yucel & Baskin, 2004).

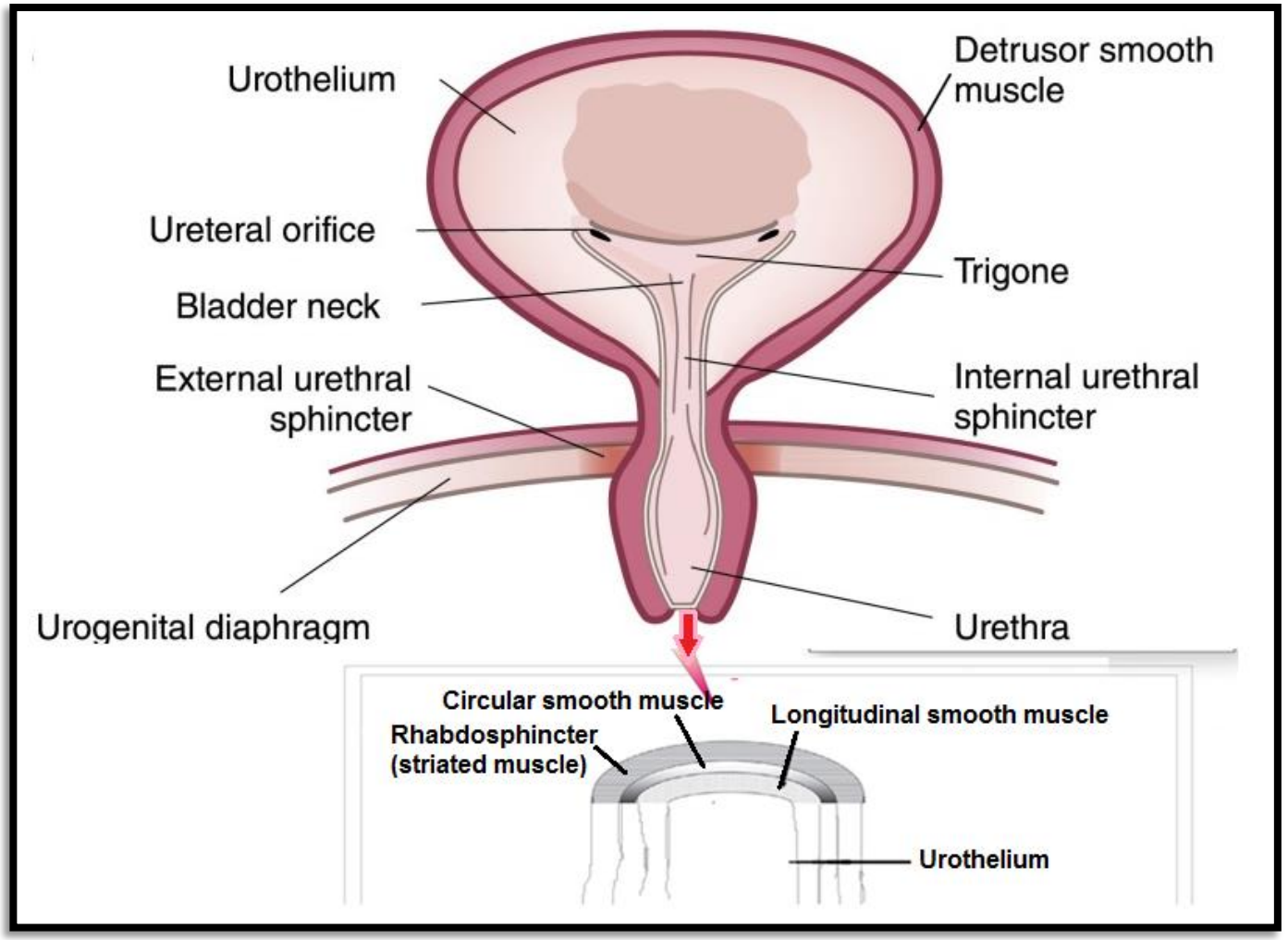


Figure 1.2 The topographical layers of the bladder and urethra. The bladder has three layers; the transitional epithelium/lamina propria, the detrusor muscle and the adventitial layer, which extends to form the layers of the urethra. The urethra itself is composed of the longitudinal and the circular smooth muscle, extensions of the bladder detrusor. Distally, the urethra is encircled by a rhabdosphincter (external urethral sphincter). Modified from (Canda et al., 2008; Hill, 2015).

The male striated muscle extends from the base of the bladder and the anterior aspect of the prostate to the full length of the membranous urethra (Figure 1.1A). The prostate is a male accessory sex gland found only in mammals that functions to produce a major fraction of seminal fluid. The human prostate sits at the base of the bladder and encircles the urethra. In some species e.g. mouse the prostate is multi-lobed (Hayashi et al., 1991). However, men have a prostate without exterior lobation that contains distinct glandular regions, including a peripheral zone, a central zone, a transition zone, and a non-glandular anterior fibromuscular stroma region. The prostate is composed of multiple glands in each of these "zones" with ductal conduits to the urethra. The prostate epithelial cell compartment is comprised of two cell layers, consisting of basal and terminally differentiated luminal cells. The luminal cells secrete prostatic secretions into the luminal space (Lilja & Abrahamsson, 1988).

The female urethra differs from that of the male in that the female urethra lacks a prostate, and is shorter (approx. 4cm) compared to the male (approx.20cm) (Fry & Young, 2010; Kohler et al., 2008). In the female urethra, the smooth muscle sphincter at the bladder neck is not evident, and the majority of muscle bundles in this region extend slanting or longitudinally into the urethra (Yucel & Baskin, 2004). The female has a weakly striated sphincter mechanism divided into a proximal and distal striated sphincter. The proximal sphincter consists of the circular muscle that forms the outermost layer of the muscle wall, while the distal comprises of two arch-shaped straps of muscle, the compressor urethrae, and urethrovaginal sphincter. The compressor urethrae arise laterally near the ischiopubic rami while the urethrovaginal sphincter closely follows the vaginal wall (Yucel & Baskin, 2004).

1.1.1.1 Smooth muscle of the lower urinary tract

The smooth muscle of the lower urinary tract is responsible for contraction of the urinary bladder and urethra and is primarily under the control of the autonomic nervous system. It is associated with maintenance of resting tone, organ dimensions due to tonic contraction, as well as relaxation of the lower urinary tract (Andersson, 2001).

In many species, detrusor smooth muscle cells are oriented longitudinally in the outer and inner layers and circularly in the middle layer (Andersson & Arner, 2004). The human detrusor is a meshwork of muscle cells of varying size surrounded by connective tissue rich in collagen (Lepor et al., 1992). The detrusor muscle meshwork is of various sizes, often a few millimetres in diameter, and composed of several smaller sub-bundles (Brading, 1987). The orientation and interaction between the smooth muscle cells in the bladder determines how the bladder wall behaves and its shape and intravesical pressure (Andersson & Arner, 2004). The detrusor smooth muscle of the bladder extends to form the longitudinal and the circular smooth muscle of the urethra, as shown in Figure 1.2 (Li et al., 2015). The longitudinal smooth muscle of the urethra is continuous with the inner longitudinal layer of the bladder detrusor while the thicker urethral circular smooth muscle is continuous with the external detrusor muscle of the bladder (Patel & Chapple, 2008).

Sphincters of the lower urinary tract

Traditionally, the urethra has been reported to have two sphincters, the internal urethral sphincter and the external urethral sphincter, also known as the rhabdosphincter. The 'internal urethral sphincter' is however controversial. Some report that the smooth muscles lining the bladder neck and the urethra form the internal sphincter (Yucel & Baskin, 2004), whilst others report that neither the internal urethral sphincter nor external urethral sphincter is visible (Lim et al., 2013). Whilst still debatable, the internal urethral sphincter is thought to be located at the inferior end of the bladder, at the level of the bladder neck. The internal urethral sphincter surrounds the proximal urethra and is seen as a continuation of the detrusor smooth muscle. In the mid-urethra, layers of striated muscle, arranged in a circular configuration, thought to be derived from levator ani, surround the smooth muscle layer of the internal urethral sphincter (Yucel & Baskin, 2004).

Striated muscle, which is present in the distal wall of the male and female urethra, forms the external urethral sphincter. The external urethral sphincter covers the inferior side of the prostate and is located at the level of the membranous urethra in males (Karam et al., 2005a). However, the external urethral sphincter is located at the distal inferior end of the bladder in females (Hudson et al., 2002). Unlike the internal urethral sphincter, the external urethral sphincter is composed of skeletal muscle; therefore, it is voluntarily controlled. The urethral

striated muscles are anatomically and functionally quite distinct from the striated skeletal muscle of the pelvic floor. They are attached to the pelvic floor muscles and each other by connective tissue but do not attach directly to pelvic floor bone.

The striated muscles of the urethra are both slow-twitch and fast-twitch. These slow twitch fibres are slower to fatigue and are responsible for maintenance of tone and the resting urethral pressure profile (Stafford et al., 2012). The fast twitch fibres come into play during transient events which increase the abdominal pressure abruptly e.g. a cough. Any activities that cause abdominal compressions, such as coughing or exercise, produce dramatic increases in the number of units firing in fast twitch fibres (Stafford et al., 2012). In the male, the rhabdosphincter consists of 35% fast-twitch and 65% slow twitch fibres (Ho et al., 1998). However, the female rhabdosphincter consists of 13% fast-twitch fibres and 87% slow twitch fibres (Padykula & Gauthier, 1970).

1.1.1.2 Urothelium of the lower urinary tract

A transitional epithelium (urothelium) lines the entire lower urinary tract (Achtstatter et al., 1985), including the renal pelvis, ureters, bladder, upper urethra and glandular ducts of the prostate (Khandelwal et al., 2009). It forms the interface between the urinary space and the underlying vasculature, connective, nervous, and muscular tissues (Khandelwal et al., 2009). The urothelium differs from one part of the lower urinary tract to another. A distinct difference in morphology of ureteral and bladder urothelial cells exist. However, there seems to be no apparent difference between the urothelium of the trigone compared with the detrusor (Jost et al., 1989; Liang et al., 2005). Therefore, the urothelium has been classified into three lineages; (I) ureter/renal pelvis (II) bladder neck/proximal urethra and (III) bladder/trigone urothelium (Liang et al., 2005).

The outer layer of the bladder urothelium is made up of hexagonal umbrella cells lined with glycosaminoglycan/mucin, uroplakins, and cytokeratins (Buckley et al., 1996; 2000). The intermediate layer is made up of pear-shaped cells, while the cells of the basal layer are mononucleated (Figure 1.3). The umbrella cells of the bladder aid in increasing surface area. This process occurs during filling, in a process achieved by exocytosis and endocytosis (Kreft et al., 2009; Truschel et al., 2002). Likewise, the tight junctions between these cells form a

barrier which prevents ions and solutes from moving through the urothelium. Moreover, uroplakin protein in the bladder umbrella cells of the apical layer reduces infiltration of small molecules such as water, urea, and protons through the urothelium, while the mucin layer of the umbrella cells protects the lower urinary tract from microbial adherence and infection (Mulvey et al., 1998).

The urothelium of the proximal urethra transitions to a stratified or columnar epithelium accompanied by a lack of urothelial-specific differentiation markers. The epithelial lining of the anterior urethra of males consists of stratified columnar epithelium except at the fossa navicularis where it becomes stratified squamous epithelium. The epithelial lining of the female urethra is transitional in the proximal one-third and non-keratinized stratified squamous in the distal two-thirds. The uroplakins are absent from the urethral urothelium (Romih et al., 2005), although there are microvilli on the apical surface of the urethral urothelium. The microvilli may play a role in increasing the surface area of the cell, participation in both sensory and transducer functions, and in fluid transport.

The lamina propria also referred to as the sub-urothelium lies between the basement membrane of the urothelium and the detrusor muscularis (Figure 1.3). It is composed of an extracellular matrix containing several types of cells, including fibroblasts, adipocytes, interstitial cells (IC), and afferent and efferent nerve endings (Gevaert et al., 2011; Gosling & Dixon, 1974; Wiseman et al., 2003). The lamina propria also contains a rich vascular network, lymphatic vessels, elastic fibres, and smooth muscle fascicles (muscularis mucosae) (Dixon & Gosling, 1983; Paner et al., 2007; 2009).

The role of the urothelium as a transducer of signals is also apparent in its expression of sensory receptors including purinergic, adrenergic, nicotinic, muscarinic and oestrogen receptors (Figure 1.4) (Beckel et al., 2006; Bschleipfer et ., 2007; Moro et al., 2013; Shabir et al., 2013; Teng et al., 2008). These receptors enable the urothelium to respond to sensory inputs (physical and chemical) from a variety of sources. The urothelium can respond to a wide range of mechanical stresses during bladder filling and emptying including bladder pressure, tension in the urothelium or bladder wall, torsion, geometrical tension, movement of visceral organs and even urine tonicity. Afferent and autonomic efferent nerves are located

near the urothelium, and can be activated by a variety of transmitters and mediators released in part by the urothelium (Birder et al., 2002a; Gabella & Davis, 1998; Grol et al., 2008).

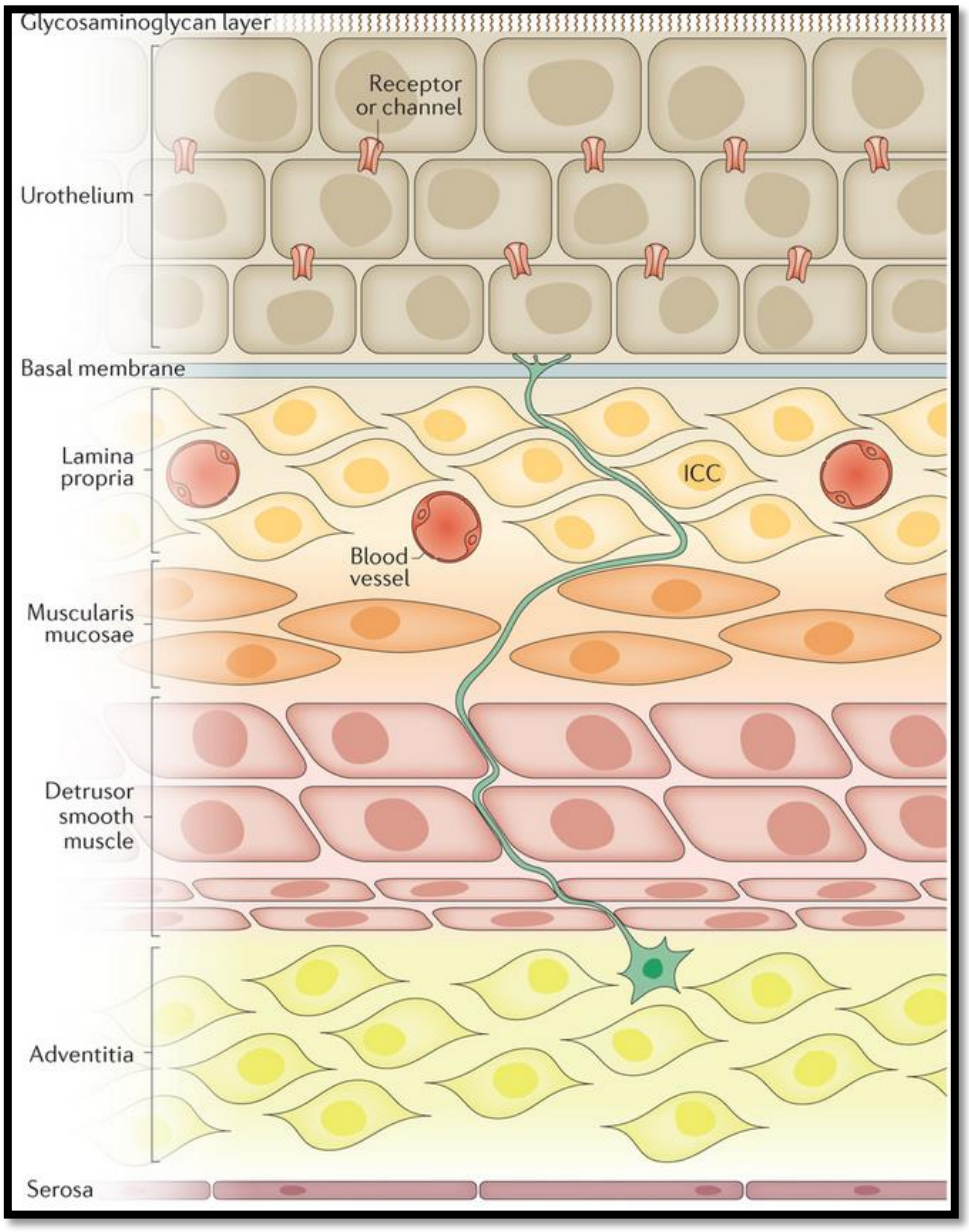


Figure 1.3 The diagrammatic representation of the urothelium layers and components, smooth muscle and serosa. ICC= interstitial cells of Cajal. Green structure= neurone. Modified from Merrill et al., 2016.

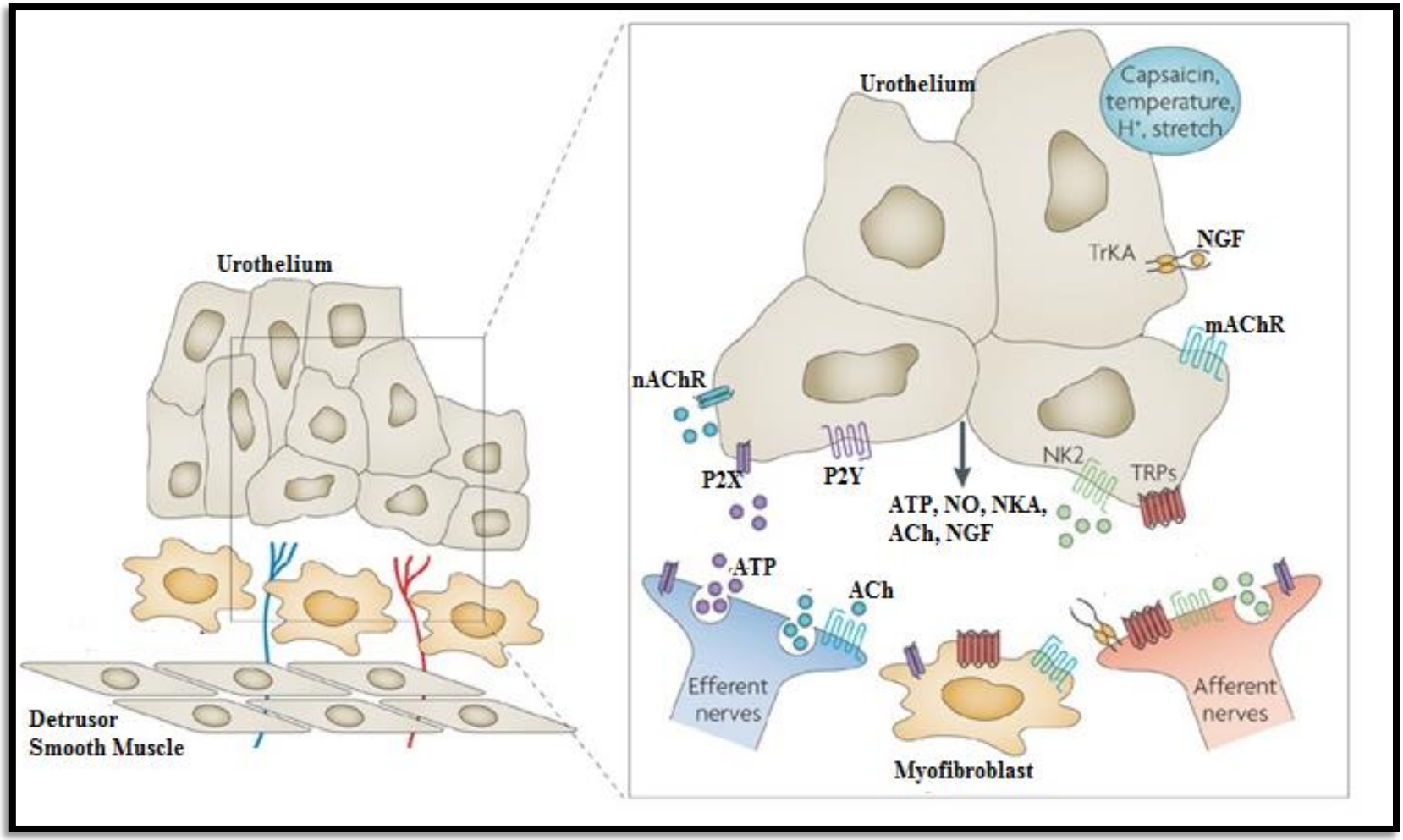


Figure 1.4 The urothelium is not just a barrier. The urothelium expresses receptors and serves as a transducer; communicating physical and mechanical stimuli to the underlying cells via secretion of chemical mediators such as neurotransmitters and cytokines. ACh= acetylcholine, mAChR= muscarinic acetylcholine receptor, nAChR= nicotinic acetylcholine receptor, NO=nitric oxide, NKA=Neurokinin A, NGF= Nerve growth factors. Modified from (Birder, 2010).

1.1.1.3 Interstitial cells

In recent years, cells resembling the interstitial cells of Cajal (ICC) of the gastrointestinal tract have been discovered in the lower urinary tract (Smet et al., 1996). In the gastrointestinal tract, these cells act as pacemakers and drive peristaltic activity (Nakagama et al., 2005). Interstitial cells of Cajal can be identified with antibodies raised against the Kit receptor (cluster of differentiation [CD117] of the receptor tyrosine kinase) (Gevaert et al., 2012). Moreover, ICC has been identified with antibodies to vimentin, and more recently, platelet-derived growth factor receptor-α (Koh et al., 2012; Monaghan et al., 2012). Kit (CD117) is a member of the family of CD cell surface molecules, which perform some important roles in the development of the immune system and physiology of various cells, including gastrointestinal ICCs. Kit (CD117) is also a proto-oncogene, and its mutation or overexpression can lead to the development of cancers in some organs including the ovaries, testes, skin, gastrointestinal tract, blood and the kidney (Ahmed & Youssif, 2009; Heinrich et al., 2003).

Kit-positive interstitial cells were found within the smooth muscle layers of the rabbit urethra, in association with nerves, on the edge of smooth muscle bundles and in the inner bundle spaces. The contact with neuronal nitric oxide synthase (NOS)-containing neurones implies participation in the nitrergic inhibitory neurotransmission of the urethra (Lyons et al., 2007). Much evidence suggests that the urothelium together with suburothelial ICC (myofibroblasts) may serve as a mechanosensor in the bladder (Sui et al., 2008).

Interstitial cells of Cajal-like cells (ICC-LCs) may play a major role in the nerve-mediated modulation of detrusor smooth muscle excitability (Davidson & McCloskey, 2005; Smet et al., 1996). Interstitial cells of Cajal-like cells may also be involved in neuromuscular transmission, and thus changes in ICC-LC numbers may account for increased excitability of detrusor smooth muscle in overactive bladders. Generation of spontaneous transient depolarizations occurs in urethral ICC-LCs, while isolated single smooth muscle cells are electrically quiescent, indicating that ICC-LCs in the urethra may have a genuine pacemaker role (Sergeant et al., 2000). Urethra ICC-LCs may randomly increase excitability of urethral smooth muscle cells to maintain the tone of urethral smooth muscles (Hashitani & Suzuki, 2007). The precise role of these cells however remains unclear.

1.1.2 Innervation and function of the lower urinary tract

The lower urinary tract receives an efferent innervation from the thoracic and lumbosacral segments of the spinal cord. Efferent axons are carried in three sets of peripheral nerves: sacral parasympathetic (pelvic nerves), thoracolumbar sympathetic (hypogastric nerves), and sacral somatic nerves (pudendal nerves) (Kluck, 1980; Thor et al., 1989). Preganglionic axons carrying information from the spinal cord to the bladder and urethra synapse with autonomic ganglion cells. Acetylcholine acting on nicotinic acetylcholine receptors mediates synaptic transmission in all ganglia (Park et al., 2006). Sympathetic preganglionic pathways that arise from the T_{11}–L_2 spinal segments pass to the sympathetic chain ganglia and then to prevertebral ganglia in the superior hypogastric and pelvic plexus and also to short adrenergic neurones in the bladder and urethra. Sympathetic postganglionic nerves that release noradrenaline provide an excitatory input to smooth muscle of the urethra and bladder base, and inhibitory input to smooth muscle in the body of the bladder (Figure 1.5).

Sacral parasympathetic efferent nerves from the S_2-S_4 level of the sacral spinal cord (Figure 1.5) provide an excitatory cholinergic input to the bladder smooth muscle and a nitric oxide (NO)-mediated inhibitory input to the urethral smooth muscle. Also, somatic cholinergic nerves, which originate from the Onuf nucleus in the sacral spinal cord (S_2-S_4 level) (Figure 1.5) excite the skeletal smooth muscle at the distal urethra (Thor et al., 1989).

1.1.2.1 Sympathetic innervation

The catecholamine noradrenaline is synthesised from tyrosine, stored in synaptic vesicles and released by sympathetic adrenergic nerves, activating the adrenoceptors. The synaptic reuptake and degradation of noradrenaline terminate its action. Noradrenaline mediates contraction of the bladder neck/ proximal urethra via adrenoceptors (Kedia et al., 2013).

Adrenoceptors in the lower urinary tract

Adrenoceptors (ARs) belong to the large family of G-protein-coupled receptors (GPCRs), also known as seven-transmembrane receptors (7-TMRs), which transduce extracellular stimuli into cellular responses. Adrenoceptors were initially subdivided into two main types,

α- and β-, based on the rank orders of the potency of noradrenaline, adrenaline and isoprenaline, as well as the physiological outcome of the response (contraction vs. relaxation) (Ahlquist, 1948).

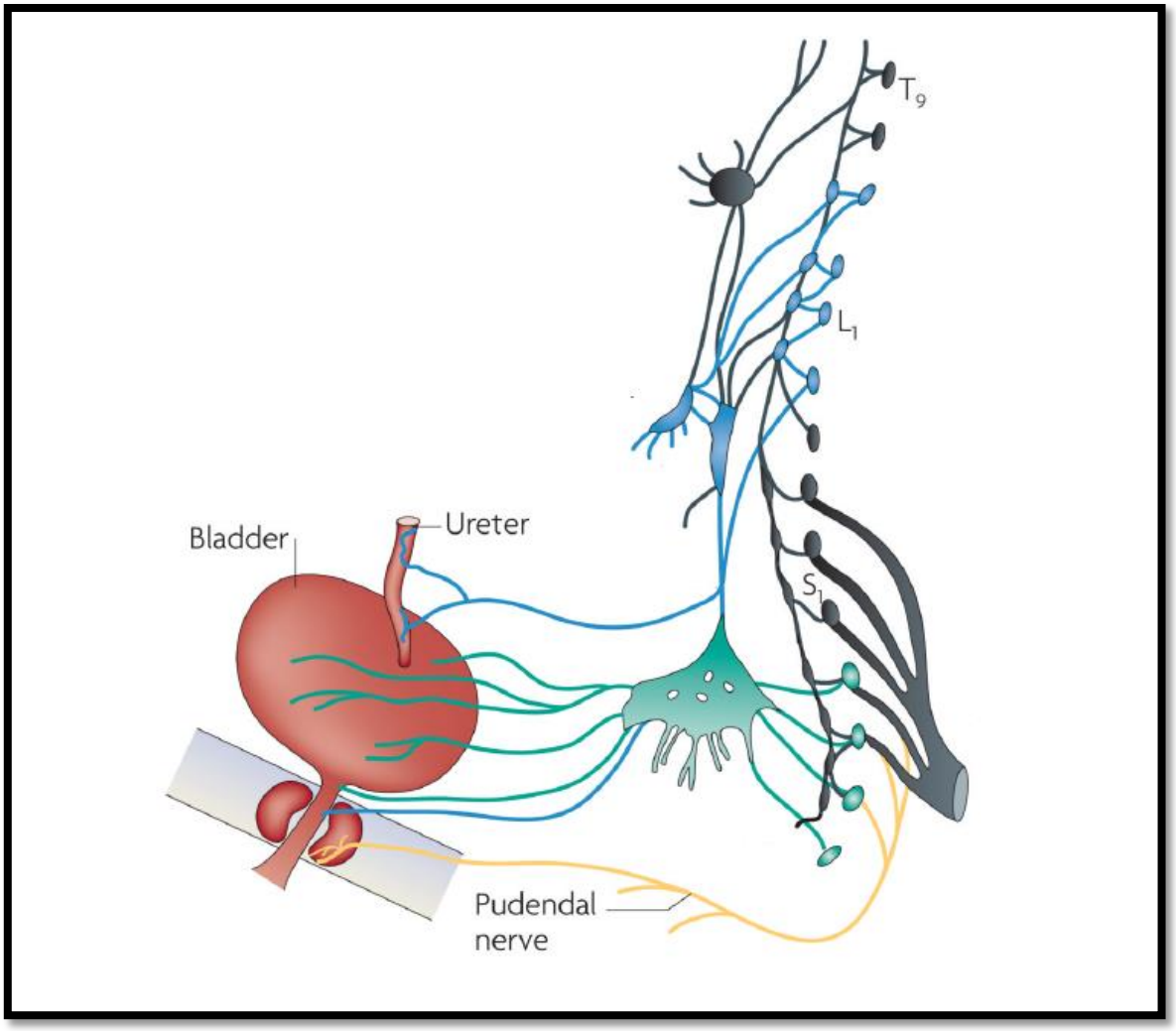


Figure 1.5 Innervation of the female lower urinary tract. The hypogastric sympathetic nerves (blue), pelvic parasympathetic nerves (green) and somatic pudendal nerves (orange) innervate the female lower urinary tract. The sympathetic innervation originates from T_{11}-L_2 of the thoracolumbar region of the spinal cord while the pelvic parasympathetic nerves originate from the S_2-S_4 of the sacral spinal cord. Moreover, the somatic pudendal nerves also originate from the S_2-S_4 region of the sacral spinal cord. Adapted from (Fowler et al., 2008).

With the discovery of new synthetic and more selective ligands, new receptor subtypes were identified within each of the two groups. Beta-ARs include the β_1, β_2, and β_3- subtypes

(Emorine et al., 1989; Lands et al., 1967), and the introduction of molecular cloning confirmed the existence of these genetically and pharmacologically distinct subtypes of β-AR. The technique also allowed a final classification of the α_1- subgroup into α_{1A}-, α_{1B}- and α_{1D}-ARs (Ford et al., 1994) and α_2- into α_{2A}-, α_{2B}- and α_{2C}-ARs (Bylund, 1992; Bylund et al., 1992). The three subtypes of the α_1-AR have been identified corresponding to the ADRA1A (α_{1a}), ADRA1B (α_{1b}), and ADRA1D (α_{1d}) genes. Pharmacological profiles of these α_1-AR subtypes, all typified by a high affinity (subnanomolar KD) for prazosin (a prototypic, selective α_1-AR antagonist) have been verified for the recombinantly expressed receptors, which reflect the same phenotype observed for these subtypes in intact tissues (Cotecchia et al., 1988; Lomasney et al., 1991; Perez et al., 1991; Schwinn et al., 1990; 1995).

The major AR isoforms present in the human bladder are α_1-ARs and β_3-ARs (Figure 1.6). However, the expression of α-ARs, as determined at the mRNA and protein levels, is very low compared with β-ARs (Nomiya & Yamaguchi, 2003). The direct effects of α_1-AR stimulation on detrusor contraction are rather weak, amounting for approximately 10%–40% of the contractile force that results from muscarinic acetylcholine receptor (mAChR) stimulation. $Alpha_1$-AR-mediated function is prominent near the bladder outlet and maintains outlet resistance (Michel & Barendrecht, 2008). Therefore, α_1-ARs appear to play only a minor functional role in the detrusor.

The α_1-Adrenoceptors: The presence of α_1-ARs in the urethra has been assessed at the mRNA level, protein level (Yono et al., 2004) and pharmacologically characterised in pigs (Bagot & Chess-Williams, 2006), rat (Fukata & Yamazaki, 2016) and human (Yoshiki et al., 2013). Contractions mediated through α_1-ARs have been reported in the urethral tissue of animals and man (Al-Noah et al., 2014; Kedia et al., 2013; Yoshiki et al., 2013). The entire length of isolated female human urethral strips contracts to noradrenaline, but not clonidine (α_2-adrenoceptor agonist) with a peak in the mid- to proximal urethra, confirming that α_1-ARs mediate urethral contraction (Taki et al., 1999). Furthermore, studies in animals and human urethral tissues have detected a rank order of abundance of α_{1A}-ARs > α_{1B}-ARs ≥ α_{1D}-ARs (Yono et al., 2004).

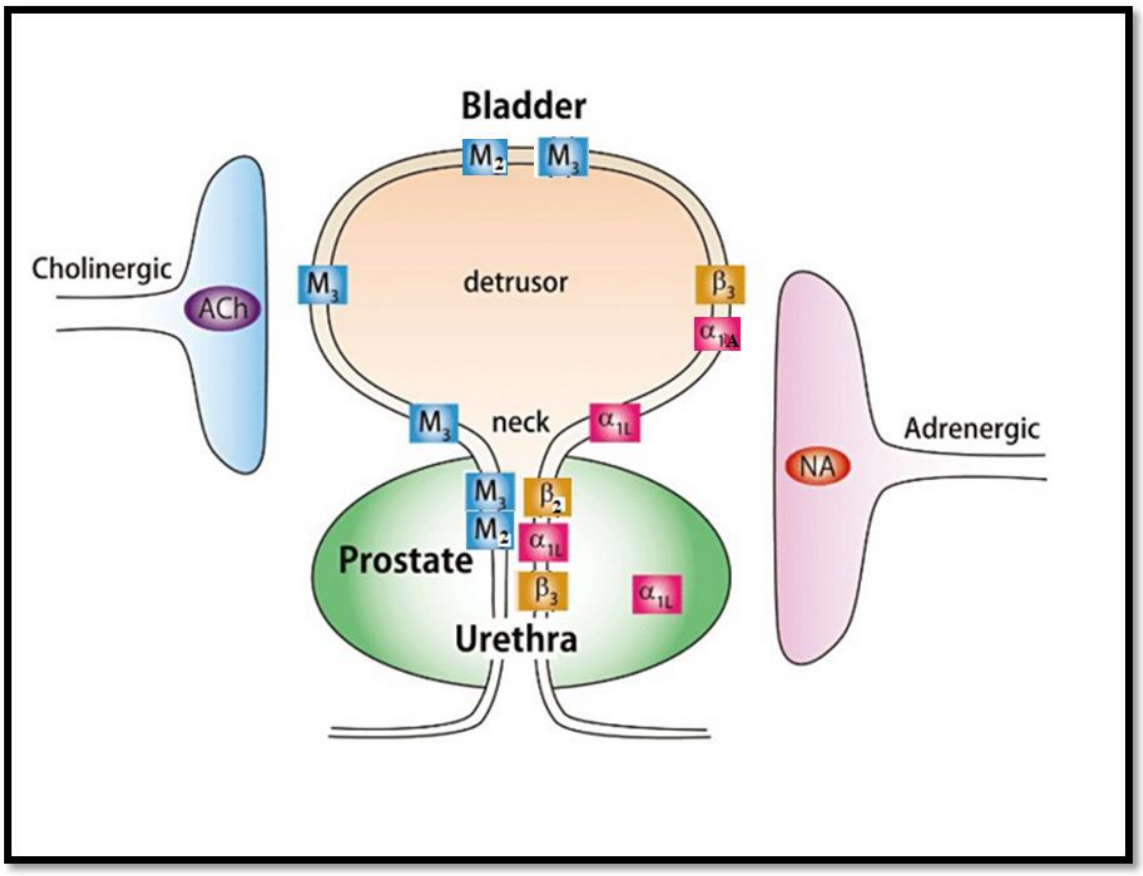


Figure 1.6 Adrenoceptor and muscarinic receptor expression in the lower urinary tract. The human bladder expresses α_1 and β-adrenoceptors as well as muscarinic receptors (M). Noradrenaline stimulates contraction of urethral and bladder neck smooth muscle via activation of the α_{1A}-adrenoceptors and causes relaxation of detrusor via activation of the β_2-adrenoceptors and β_3-adrenoceptors. The detrusor expresses both M_3 and M_2. However, the M_3 receptors mediate the detrusor contraction to acetylcholine (Yamanishi et al., 2000). α_{1L}-ARs are a functional phenotype of the α_{1A}-ARs. ACh= acetylcholine & NA= noradrenaline. Modified from (Nishimune et al., 2012).

The α_1-ARs are coupled to pertussis toxin insensitive G proteins ($G_{q/11}$), and their effector is tissue and species specific (Wu et al., 1992). Activation of G_q activates phospholipase C which releases inositol 1, 4, 5-tris-phosphate and diacylglycerol from phosphatidyl inositol-4, 5-bisphosphate hydrolysis. Moreover, activation of the α_1-ARs has been associated with activation of other effector proteins such as phospholipase A_2, phospholipase D and Ca^{2+} channels (Ruan et al.,1998; Xing & Insel et al., 1996).

The α_{1A}-ARs of isolated tissues from prostate gland (Hiraoka et al., 1995), urethra (Bagot & Chess-Williams, 2006; Yoshiki et al., 2013), bladder (Yoshiki et al., 2013) and iris

(Muramatsu et al., 2009) exhibit a low affinity for prazosin. This receptor is referred to as the α_{1L}-AR, reflecting its unique low affinity for prazosin (Muramatsu et al., 2009). The α_{1L}-ARs relatively low affinity in functional and binding assays for prazosin ($pK_i < 9$), might represent different receptor states between α_{1A}-AR and α_{1L}-AR (Alexander et al., 2011). This receptor also shows low affinity for 5-methylurapidil and RS-17053 (α_{1A}-antagonists), and BMY 7378 (α_{1D} -antagonist). It has high-affinity for silodosin (α_{1A}-AR antagonist) and tamsulosin (an α_{1A}-AR antagonist) (Ford et al., 1996; Muramatsu et al., 1998; Murata et al., 1999; Morishima et al., 2007; Testa et al., 1997).

The two distinct α_1-AR phenotypes (α_{1A}-ARs and α_{1L}-ARs) are known to originate from a single ADRA1A (α_{1a}) gene by an unknown mechanism. It was demonstrated with the use of a genetically modified AR knockout mouse that α_{1L}-ARs are indeed a functional phenotype of the α_{1A}-AR (Gray et al., 2008; Muramatsu et al., 2008). Gray et al., (2008) showed that α_{1L}-AR-mediated responses in mouse prostate are abolished in α_{1A}-AR knockout mouse, demonstrating that the α_{1A}-AR gene is essential to the manifestation of the prostatic α_{1L}-AR phenotype.

Early attempts to explain how the α_{1L}-AR phenotype could arise from the α_{1A}-AR gene concentrated on genetic polymorphisms and gene splicing. However, α_{1A}-ARs generated by known polymorphisms and splice variants in cell culture models all showed similar pharmacological characteristics to that of the α_{1A}-AR (Ramsay et al., 2004; Shibata et al., 1996; Suzuki et al., 2000).

In functional studies in intact tissues only α_{1L}-ARs were detected. However, after homogenization to produce membrane homogenate for radioligand binding studies, the pharmacological profile of the α_{1L}-ARs was lost (Morishima et al., 2007; 2008; Muramatsu et al., 2008; Su et al., 2008a; Suzuki et al., 2000) and an 'interacting protein' theory to explain the generation of α_{1L}-ARs from α_{1A}-ARs was postulated. Nishimune et al., (2010a) suggested that this discrepancy was caused by the homogenization process disrupting the cell membrane and thus separating α_{1A}-ARs from the putative 'interacting protein'.

In support of the 'interacting protein' theory, Nishimune et al., (2010b) identified cysteine-rich epidermal growth factor-like domain 1α (CRELD1α) as a novel down-regulating protein

and therefore a protein that interacts with the α_{1A}-ARs. Nishimune et al., (2010b) hypothesised that the CRELD1α protein interaction with α_{1A}-AR could generate the α_{1L}-AR phenotype. Transfection of α_{1A}-AR cDNA alone yielded Chinese hamster ovary (CHO) cells expressing α_{1A}-ARs having a predominant high-affinity site for prazosin, with a low proportion (<10%) of prazosin-low affinity sites (α_{1L}-ARs). However, over-expression of CRELD1α reduced α_{1A}-ARs expression, yielding α_{1L}-ARs -dominant cells expressing a high proportion (50%) of the α_{1L}-ARs phenotype.

The α_2-Adrenoceptors: Radioligand binding experiments have detected α_2-ARs in the detrusor of rabbits (Latifpour et al., 1990) and human (Goepel et al., 1997). The function of α_2-ARs in the bladder depends on the α_2-ARs stimulated (the central or peripheral α_2-ARs) (Michel & Vrydag, 2006). Moreover, in vivo, the effect of α_2-AR agonists depends on the use of anaesthetized or conscious animals. Clonidine, oxymetazoline and tizanidine (α_2-AR agonists) caused bladder hyperactivity in the conscious male rat, which was prevented by the selective α_2-AR antagonist, idazoxan (Kontani et al., 2000). Likewise, Jeong & Lee, (2000) reported in a cystometry experiment in anaesthetised rats that clonidine markedly increased bladder basal pressure and frequency, whereas micturition volume and bladder capacity was not affected. However, intravenous dexmedetomidine (α_2-AR agonist) significantly decreased the maximum bladder pressure and urinary flow rate, and the amplitude of rhabdosphincter electromyography in the anaesthetized rat (Streng et al., 2010). Dexmedetomidine also abolished the intraluminal pressure high-frequency oscillations, usually observed during rat voiding.

Urethral α_2-ARs have also been identified in radioligand-binding studies in rabbits (Latifpour et al., 1990). Larsson et al., (1986) found that the density of α_2-ARs increased from the proximal to the distal urethra, which appeared to belong largely, if not exclusively, to the α_{2A}-subtype (Latifpour et al., 1990). The α_2-AR can elicit urethral contraction in rabbits (Andersson et al., 1984) and horses (Garcia-Sacristan et al., 1984). Moreover, clonidine increased intra-urethral pressure via α_2-ARs in the dog (Shapiro et al., 1987).

The β-adrenoceptors: Expression of all three β-AR subtypes has been found both at the mRNA and at the protein level in the human bladder (Nomiya & Yamaguchi, 2003). Cellular, in vitro and subsequent in vivo studies, have shown the predominant β-AR in human bladder

tissue to be β_3-AR (Igawa et al., 2001). The β_3-AR isoform in the human bladder accounts for >95% of β-ARs. However, both β_3 and β_2-ARs contribute to relaxation responses of the rat bladder (Oshita et al., 1997). Pig detrusor smooth muscle also has a similar population of β-AR subtypes, with β_3-ARs predominating and a minor population of β_2 -ARs (Yamanishi et al., 2002b).

The β_3-ARs largely mediate isoprenaline-induced bladder relaxation in humans, whilst in rats both β_2 and β_3-ARs are involved (Igawa et al., 1999; Michel et al., 2011; Takeda et al., 1999). Isoprenaline induced relaxation via β_2-ARs and β_3-ARs in the pig urethra, in spite of β_3-ARs predominating (Yamanishi et al., 2002c: 2003b). Takeda et al., (2003) also showed that the relaxations in canine and rat urethra were predominantly mediated by β_2-ARs and β_3-ARs, with a greater role for β_2-ARs.

The canonical β-AR signalling pathway involves G_s-protein-mediated adenylyl cyclase activation, cyclic AMP elevation and subsequent protein kinase A activation (Frazier et al., 2008). Moreover, there is evidence of cyclic AMP-dependent, as well as independent effects of β-AR stimulation (Frazier et al., 2008). Compelling evidence suggested that β-AR can also stimulate large-conductance Ca^{2+}-activated K^+ channels, for example in the guinea pig (Petkov and Nelson, 2005), rat (Hristov et al., 2008; Uchida et al., 2005), mouse (Brown et al., 2008) and human bladder (Afeli et al., 2013). Combined administration of inhibitors of the cyclic AMP/protein kinase A and the large-conductance Ca^{2+}-activated K^+ channels pathway inhibited β-AR-mediated bladder relaxation by less than half (Frazier et al., 2005), suggesting that other pathways might also be involved in β-AR-mediated response. Recently, Cernecka et al., (2015) showed in the rat and human bladder that the extent of large-conductance Ca^{2+}-activated K^+ channels and Rho kinase involvement in relaxation induced by β-AR depends on pre-contractile stimulus and species.

1.1.2.2 Parasympathetic innervation

The main contractile transmitter in the human bladder is acetylcholine (Creed et al., 1994). It is released from postganglionic efferent cholinergic (parasympathetic) nerves, acts on mAChRs, and produces the contraction that empties the bladder (Zagorodnyuk et al., 2009; Wang et al., 1995). Acetylcholinesterase-positive nerves are also present in the urethral tissue

of the pig (Crowe & Burnstock, 1989) and human (Kluck, 1980), and acetylcholine induces contraction in the proximal urethra (Taki et al., 1999). However, Kedia et al., (2013) reported a minor contractile effect of acetylcholine in the human male urethra. Acetylcholine is also associated with contraction of the urethral rhabdosphincter, by activation of the nicotinic acetylcholine receptor (Blaivas, 1982; Thor et al., 1989).

Muscarinic receptors

The bladder, bladder base and urethra express mAChR subtypes (M_1–M_5) (Figure 1.6) (Sigala et al., 2002; Tyagi et al., 2006). In the human detrusor, 70 percent of the total mAChR population are the M_2 subtype, 20 percent are M_3 subtype while 10 percent are M_1 subtype (Mansfield et al., 2005). In rats, pigs and humans, the ratio between M_2 and M_3 receptors in binding studies was estimated as 9:1, 3:1 and 3:1, respectively (Wang et al., 1995; Mansfield et al., 2005; Yamanishi et al., 2002a). Although there was a predominance of M_2 receptors in the detrusor, the contraction was mediated mainly by the M_3 receptors in rat (Hegde et al., 1997), pig (Sellers et al., 2000; Yamanishi et al., 2002a) and human (Fetscher et al., 2002). In the human detrusor, muscarinic agonist-induced contraction largely depends on Ca^{2+} entry through nifedipine-sensitive channels and activation of the Rho kinase pathway (Schneider et al., 2004b).

In the human urethra, mAChR activation contracts the proximal urethra and bladder neck (Taki et al., 1999). Similar, in the pig mAChRs (M_2 and M_3) seem to mediate contraction of the bladder base and urethra (Yamanishi et al., 2002a), although, M_3 receptors are mainly responsible for contraction. In rabbits (Nagahama et al., 1998), rat (Kakizaki et al., 1997) and hamster (Pinna et al., 1996) muscarinic stimulation caused urethral relaxation. However, the cholinergic response in the rabbit urethra is site dependent; mAChR-mediated contraction of rabbit proximal urethra and relaxed the distal urethra through the release and actions of NO (Nagahama et al., 1998).

Co-transmission in the lower urinary tract

Atropine-resistant contractile responses of the urinary bladder following stimulation of parasympathetic nerves have been shown to be due to non-cholinergic, non-adrenergic transmission in animals and human detrusor (Bicer et al., 2012). In the normal human bladder

this component amounts to only a few percent, whereas in for example rodent bladder, the atropine-resistant component can exceed 50% (Brading & Williams, 1990; Igawa et al., 1993). The atropine-resistant component in the bladder is purinergic and ATP is co-released with acetylcholine in the bladder (Tsai et al., 2012). Purinergic receptors respond to nucleosides and nucleotides including adenosine, ADP, ATP, UTP, and UDP. The P1 and P2 receptors are the two classes of purinergic receptors. The P1 receptors respond to adenosine while P2 receptors respond to nucleotides. P2 receptors are classified as ligand-gated ion channels (P2X) or GPCR (P2Y) (Fredholm et al., 1994). There are currently seven P2X subunits ($P2X_{1–7}$) that may arrange as heteromeric or homomeric ligand-gated ion channels and eight metabotropic P2Y subunits ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) that may couple to G_s, G_i, or G_q (Abbracchio et al., 2006). The P2 receptors are expressed throughout the urinary bladder detrusor (Vial & Evans, 2000). The primary purinergic receptor present on the detrusor is P2X, as confirmed by organ bath experiments, immunostaining (Lee et al., 2000) and $P2X_1$ knockout mouse (Vial & Evans, 2000). $P2X_{1–6}$ receptors have been demonstrated in rodent urinary bladder smooth muscle cells (Dutton et al., 1999). Moreover, work by Yu et al., (2013) confirmed the functional evidence for a P2Y (P2Y6) in the mouse detrusor. In some species ATP produces a dual effect (contraction and relaxation) on detrusor smooth muscle via activation of P2X and P2Y receptors respectively (Bolego et al., 1995; McMurray et al., 1998).

ATP released from inhibitory nerves induces relaxation of the urethra of the hamster (Pinna et al., 1998) and pig (Werkstrom & Andersson, 2005). ATP causes only relaxation of the urethra via P2Y receptors, in several species including pig (Hernandez et al., 2009) and hamster (Pinna et al., 1998).

Afferent and efferent nerves also express NOS (Vizzard et al., 1994; 1995a; 1996). Nitric oxide synthase-containing nerve fibres normally occur in the bladder, and NO thus seems to have a physiological impact on efferent and afferent neurotransmission in the normal bladder (Ehren et al., 1994). The absence of NO causes bladder overactivity, possibly via modulation of the threshold for afferent nerve firing (Ozawa et al., 1999; Pandita et al., 2000). The expression of NOS-containing nerves is greatest in the outlet region of the bladder (Persson et al., 1993; Dixon & Jen, 1995). The urethra also contains a dense network of NO expressing nerves, and the presence of NOS has been confirmed by constitutive NOS

immunohistochemistry, NADPH diaphorase immunohistochemistry and inhibition of NOS in a functional organ bath experiment (Garcia-Pascual et al., 1996). The urethra, bladder neck/trigone smooth muscle was positive for NADPH-diaphorase/NOS in the rat (Radziszewski et al., 1996), sheep (Garcia-Pascual et al., 1996), pig (Persson et al., 1993) and human (Leone et al., 1994). Likewise, neuronal nitric oxide synthase (nNOS) has been reported in the rhabdosphincter of the human (Ho et al., 2003) and sheep (GonzÁLez-Soriano et al., 2003). Thus NO modulates the lower urinary tract responses.

In the clinical setting, the NO pathway has been targeted via the use of phosphodiesterase inhibitors, such as sildenafil, which result in elevated levels of intracellular cyclic GMP and enhanced relaxation of the bladder neck and urethra (Uckert et al., 2008). In one study, a group of healthy male volunteers, given a NO donor sublingually and studied urodynamically, exhibited lowered bladder outlet resistance characterised by reduced detrusor pressure during voiding and a lower maximal flow rate (Muntener et al., 2006). Thus NO relaxes the bladder neck and urethra.

1.1.2.3 Sensory innervation of the lower urinary tract

Afferent axons in the pelvic, hypogastric, pudendal, and levator ani nerves transmit information from the lower urinary tract and pelvic floor to second-order neurones in the lumbosacral spinal cord (De Groat, 1986; Janig & Morrison, 1986). The small myelinated (Aδ) and unmyelinated C-fibres are the two populations of pelvic nerve afferents that innervate the bladder and urethra. Aδ fibres, located primarily in the detrusor smooth muscle layer, respond to detrusor stretching during bladder filling and convey fullness sensations (Zagorodnyuk et al., 2006; 2007). Aδ mechanoreceptor afferents identified in the pelvic nerve have conduction velocities ranging between 2.5 and 15 m/s (Habler et al., 1993). They are silent when the bladder is empty but during slow filling of the bladder display a graded increase in discharge frequency at intravesical pressures below 25 mmHg (Bruns et al., 2011). Electrophysiologic studies in cats revealed that the normal micturition reflex is triggered by myelinated Aδ -fibre afferents (Habler et al., 1990).

Unmyelinated sensory C-fibres are more widespread and reside in the detrusor muscle, close to the urothelium in the lamina propria and directly adjacent to urothelial cells (Wakabayashi

et al., 1993). C-fibres carry information on bladder volume changes (Morrison, 1999). Volume receptor afferents are mainly C fibres that discharge during normal bladder distention but with higher thresholds than Aδ in-series tension receptors (Habler et al., 1990). Various neurotransmitters and chemical mediators released by the detrusor and the urothelium activate the C-fibres. Bladder C-afferents in cats are mechano-insensitive ("silent C-fibres") (Habler et al., 1990). Many of these afferents are nociceptive and respond to cold stimuli or chemical/ noxious stimuli such as high potassium, low pH, high osmolality, and irritants such as capsaicin and turpentine oil (Habler et al., 1990). The C-fibre afferents express various receptors including transient receptor potential vanilloid-1 and the capsaicin receptor (Avelino et al., 2002). These fibres also express the transient receptor potential ankyrin 1 receptor (Streng et al., 2008), mAChR, endothelin receptor, and purinergic receptors ($P2X_2$, $P2X_3$, P2Y) that can be activated by ATP (Cockayne et al., 2005; Munoz et al., 2012).

Immunohistochemical studies have revealed that the bladder afferent neurones contain various neuropeptides, such as substance P, calcitonin gene-related peptide, pituitary adenylate cyclase-activating polypeptide and vasoactive intestinal peptide. The neurones also contain the putative excitatory amino acid transmitters, glutamic and aspartic acid (Keast & Stephensen, 2000; Vizzard, 2000). Peptide-containing axons are distributed throughout all layers of the bladder but are especially dense in the lamina propria just beneath the urothelium. Primary sensory fibres are extremely abundant in the rat (Avelino et al., 2002), mouse (Yu et al., 2011) and human (Yiangou et al., 2001) urinary tract. Findings have differentiated two afferent pathways, one involving the bladder urothelium, basement membrane and lamina propria, and the second involving the detrusor muscle (myogenic pathway) (Wakabayashi et al., 1993; Yoshimura et al., 2003).

Urethral afferent-mediated neural pathways also exist (Gustafson et al., 2003). The plexus of afferent nerves is most dense in the bladder neck and proximal urethra (Gabella & Davis, 1998). Urethral afferents can evoke reflexes that augment (Gustafson et al., 2003; Jung et al., 1999) or inhibit (Chang et al., 2006; Thor & Muhlhauser, 1999) reflex bladder contractions. Urethral flows consistently evoked detrusor contractions in awake ewes (Robain et al., 2001). Robain, et al., (2001) also showed that the reflex was selectively suppressed by local anaesthesia of the urethral urothelium, indicating that the effect originated from within the

urethra. Urethral anesthetisation to block activity in urethral afferents, led to incomplete emptying and the need to strain in healthy volunteers (Shafik et al., 2003). Electrical stimulation of urethral afferents also evokes reflex bladder contractions in the cat and rat, and improves bladder emptying (Chen et al., 2012; McGee & Grill, 2014). Electrical stimulation of the pudendal nerve afferents in the cat allows both inhibition and activation of the bladder, depending on the urethral location of stimulation and the frequency (Woock et al., 2009). Thus, the afferent innervation of the urethra appears tightly associated with bladder control.

Whilst Aδ fibres are present in the proximal urethra, C-fibres increase in density towards the outflow region of the urethra, have a high mechanical threshold and respond to chemical irritation of the mucosa, as well as to changes in temperature (Gabella & Davis, 1998). The positive staining for calcitonin gene-related peptide or substance P in the lamina propria and the muscular layers in all portions of the urethra signify the presence of C-fibres (Hokfelt et al., 1978; Warburton & Santer, 1994).

Afferent signalling can be modulated by stimulation of mAChRs (Daly et al., 2010) and β-ARs (Aizawa et al., 2010). Bladder afferent nerves were found to express (by RT-PCR) the mAChR subtypes M_2, M_3, and M_4 but not M_1 and M_5 (Nandigama et al., 2010). In a rat model, intravesical oxybutynin (mAChR antagonist) depressed the afferent activity recorded in the pelvic nerve (De Wachter & Wyndaele, 2003), whilst systemic administration decreased afferent activity in C-fibres after 90 minutes. In the Aδ fibres, a decrease in afferent spike rate was significant after 30 minutes administration of oxybutynin (De Laet et al., 2006). Similar effects were obtained with darifenacin (Iijima et al., 2007). The inhibitory pre-junctional mAChRs have been classified as M_4 in the human bladder (D'Agostino et al., 2000).

Likewise, systemic administration of β_3-AR agonist inhibits the mechanosensitive Aδ afferent fibre activity and prostaglandin E_2-induced C-fibre hyperactivity (Aizawa et al., 2010). Moreover, in vivo, cystometric parameters such as voiding frequency and amplitude of voiding contractions, are improved not only by the systemic administration but also by intravesical instillation of β_3-AR agonists (Kullmann et al., 2011). Data by Kullmann et al., (2011) indicated that β_3-AR activation may alter reflex voiding via the release of factors from

the urothelium that act on afferent nerves to influence bladder contractility. To our knowledge, modulation of afferent fibre activity in the urethra is not known.

1.1.2.4 Smooth muscle contraction in the lower urinary tract

Phosphorylation and dephosphorylation of the 20-kDa regulatory myosin light chain regulates contraction and relaxation of smooth muscle (Chacko et al., 1977; Deng et al., 2011). Both Ca^{2+} and Rho kinase signalling pathways modulate myosin light chain phosphorylation (Mizuno et al., 2008; Poley et al., 2008), and these pathways constitute the classical Ca^{2+} pathway and the Ca^{2+} sensitization pathways respectively.

Calcium-dependent smooth muscle contractions and relaxations

Increased intracellular Ca^{2+} ($[Ca^{2+}]_i$) is a key event in the activation of smooth muscle contraction in response to extracellular signals (Ozaki et al., 1991; Taguchi et al., 1998). Agonists elevate cytoplasmic Ca^{2+} concentration by inducing Ca^{2+} influx from the extracellular area via L-type Ca^{2+} channels and non-selective cation currents, along with stimulating Ca^{2+} release from intracellular stores (Narayanan et al., 2012). The classical pathway for phenylephrine and noradrenaline activation of α_{1A}-ARs is elevation of intracellular Ca^{2+} (Kitazawa et al., 2009; Taguchi et al., 1998). Receptor occupation activates phospholipase C, which mediates hydrolysis of phosphatidylinositol 4, 5-bisphosphate into inositol 1, 4, 5-tris-phosphate and diacylglycerol (Dupuis et al., 2008) (Figure 1.7). Inositol 1, 4, 5-tris-phosphate binds its receptors on the sarcoplasmic reticulum to trigger Ca^{2+} release. The increase in cytosolic Ca^{2+} triggers phosphorylation of myosin light chain at Ser19, in a pathway mediated by Ca^{2+}-calmodulin (Ca^{2+}-CaM)-activated myosin light chain kinase (He et al., 2008a; Isotani et al., 2004).

Relaxation of smooth muscle is induced by dephosphorylation of myosin light chain by a specific type 1 phosphatase, myosin light chain phosphatase (Shirazi et al., 1994). Termination of the contractile stimulus leads to a decrease in $[Ca^{2+}]_i$, thereby decreasing myosin light chain kinase activity. The degree of myosin light chain phosphorylation and smooth muscle tone is determined by the relative activities of myosin light chain phosphatase and myosin light chain kinase (Kitazawa & Somlyo, 1990; Kitazawa et al., 1991).

Non-adrenergic, non-cholinergic transmitters such as NO (Kitazawa et al., 2009), carbon monoxide (Zhang et al., 2015) and hydrogen sulphide (Nalli et al., 2015) induce relaxation by reducing myosin light chain phosphorylation. For example, NO induces protein kinase G activation, which results in a decrease in $[Ca^{2+}]_i$ via inhibition of phospholipase C and inositol 1,4,5-tris-phosphate formation (Hirata et al., 1990). These transmitters also induce relaxation by activation of potassium channels (K^+ channels). For example, hydrogen sulphide activates protein kinase G, producing relaxation via activation of ATP-dependent K^+ channels in the pig bladder neck (Fernandes et al., 2013).

Sources of Ca^{2+} for contractile responses: Ca^{2+} influx and efflux modulate intracellular Ca^{2+} ion concentrations via plasma membrane channels and transporters. Ca^{2+} can also enter the cytosol from the largest intracellular Ca^{2+} store, the sarcoplasmic reticulum. These channels mobilise Ca^{2+} across a Ca^{2+} concentration gradient (Harraz & Altier, 2014). In mammalian smooth muscle, Ca^{2+} entry is through voltage-gated Ca^{2+} channels, as a key process to elevate intracellular Ca^{2+} levels (Harraz & Altier, 2014). Elevated intracellular Ca^{2+} levels were proven to be consistent with action potential spike events observed in smooth muscle cells (Kyle et al., 2013). Two types of voltage-gated Ca^{2+} channels, long-lasting and transient (L-type and T-type voltage-gated Ca^{2+} channels) have been identified in the human myocyte (Sui et al., 2003).

Greater depolarisation voltages activate L-type Ca^{2+}-channels, and their activity generates the upstroke phase of the detrusor myocyte action potential allowing for a net influx of Ca^{2+} to initiate muscle contractility (Sui et al., 2003). On the other hand, T-type Ca^{2+} channels have a lower threshold of activation and are therefore active at the resting membrane potential of approximately -40 mV and could regulate smooth muscle excitability (Sui et al., 2003). Because more negative potentials activate the T-type Ca^{2+} channels, they also facilitate L-type Ca^{2+} channels opening (Sui et al., 2003).

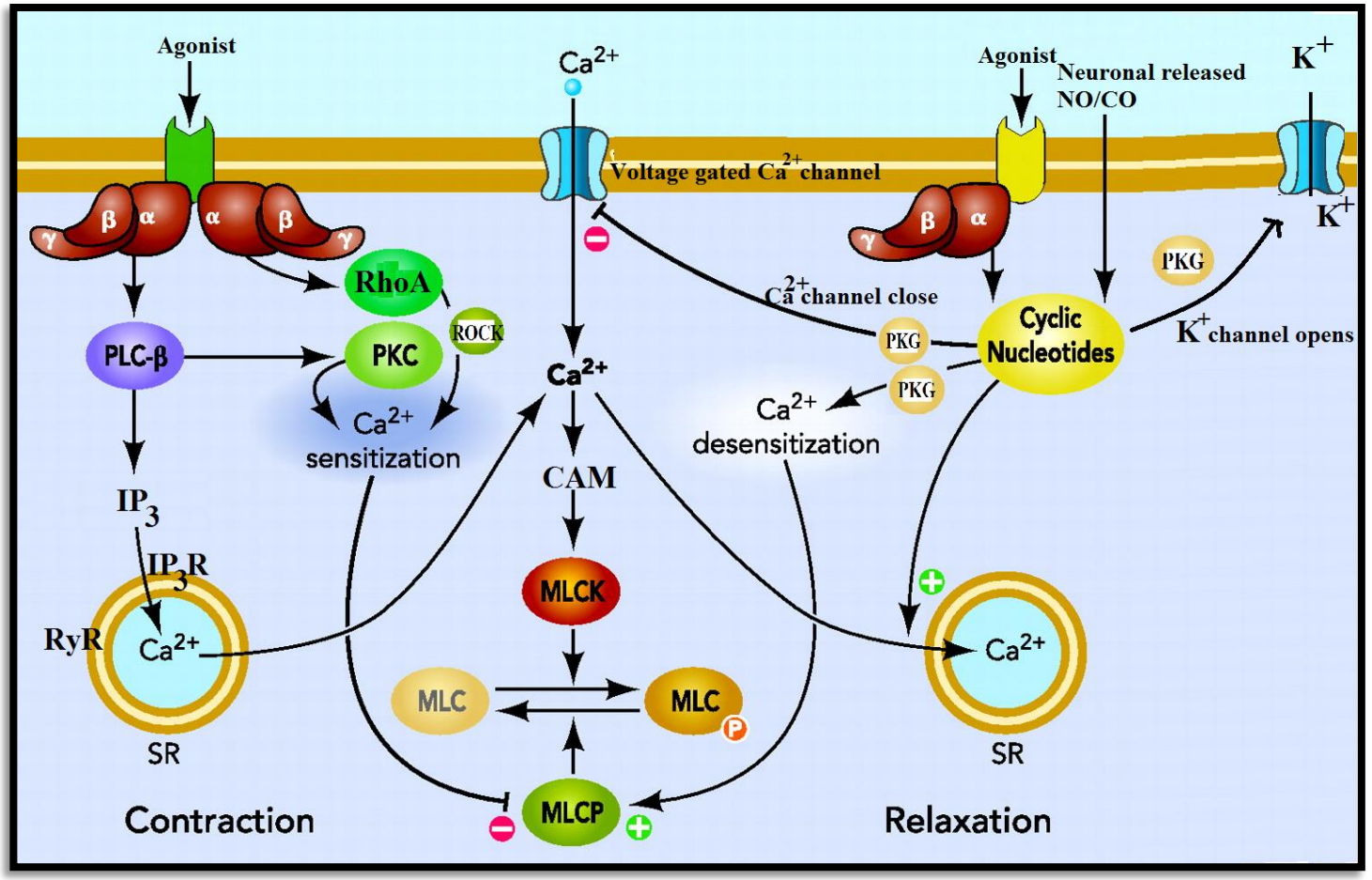


Figure 1.7 G-protein-coupled receptor (GPCR) signalling in smooth muscle, calcium sensitization, and desensitisation pathway. Activation of the GPCR induces activation of phospholipase C (PLC) which hydrolyzes phosphatidylinositol 4, 5-bisphosphate into inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol. Diacylglycerol binds Ca^{2+} to activate protein kinase C (PKC) which phosphorylates several substrates including ion channels and 17KDa PKC potentiator inhibitor protein (CPI-17). Also, IP_3 binds IP_3 receptor (IP_3R) on the sarcoplasmic reticulum (SR) to release Ca^{2+}. Released Ca^{2+} induces more Ca^{2+} release by activating the ryanodine receptors (RyR). Intracellular Ca^{2+} activates calmodulin (CAM) while active CAM activates myosin light chain kinase (MLCK) which phosphorylates myosin light chain (MLC). On the other hand, activated RhoA activates Rho-kinase (ROCK), while active Rho kinase inactivates myosin light chain phosphatase (MLCP). Hence, smooth muscle contraction. In regards to Ca^{2+} desensitisation, active protein kinase G (PKG) induces opening of several K^+ channels, the closing of Ca^{2+} channels and inhibition of RhoA. (SR: sarcoplasmic reticulum). Modified from Graham et al., 1996; Kaneko et al., 2000; Puetz et al., 2009; Carvaja et al., 2000; Surks, 2007).

Previous reports have indicated that L-type Ca^{2+} channels are mainly involved in smooth muscle contraction while the T-type Ca^{2+} channels are critical for cell proliferation (Cribbs, 2006). Recently Lu et al., (2015) showed that L-Type Ca^{2+} channels modulate the protein expression of myosin light chain, and ablation of these channels resulted in a decrease in the protein expression of myosin light chain in the mouse. L-type Ca^{2+} and T-type Ca^{2+}currents are dependent on extracellular Ca^{2+}concentration.

Ca^{2+} ions are also stored in intracellular Ca^{2+} stores that include the sarcoplasmic reticulum and endoplasmic reticulum. Store-operated Ca^{2+} channels are Ca^{2+}-permeable cation channels and are activated when the intracellular Ca^{2+} stores are depleted (Putney, 1986). The inositol 1, 4, 5-tris-phosphate pathway is the principal mechanism of store-operated Ca^{2+} channels activation (Putney & McKay, 1999). These stores are replenished through a feedback mechanism employing L-type Ca^{2+} channels (Wu et al., 2002). Ca^{2+} released from the sarcoplasmic reticulum induces further Ca^{2+} influx (Weigl et al., 2003).

Calcium sensitization and desensitisation in smooth muscle

In general, contractile agonists acting via receptors produce more tension for a given elevation of $[Ca^{2+}]_i$ than membrane depolarization alone in smooth muscle (Gerthoffer et al., 1991). This suggested that factors in addition to myosin light chain phosphorylation or changes in $[Ca^{2+}]_i$ regulate cross-bridge cycling rates in smooth muscle contraction. Since at a given $[Ca^{2+}]_i$, more force is generated, this phenomenon was termed "Ca^{2+} sensitization of the contractile apparatus", which signifies an increase in Ca^{2+} sensitivity of the contractile response (Kitazawa et al., 1991).

Ras homolog gene family, member A (RhoA) activation of Rho kinase mediates Ca^{2+} sensitization. Replacement of RhoA-bound GDP with GTP results in activation of Rho kinase leading to inhibition of myosin light chain phosphatase via phosphorylation of the myosin targeting subunit of the phosphatase (MYPT1) at Thr697 and Thr855 (rat)/ Thr696 and Thr853 (human) (Figure 1.7; Muranyi et al., 2005). However, Khasnis et al., (2014) showed that thio-phosphorylation at Thr696, but not at Thr853, inhibited the activity of recombinant myosin light chain phosphatase purified from the mammalian cell lysates. Both Thr696 and Thr853 sites underwent autodephosphorylation. The phosphorylation of Thr696 was more

stable compared with that of Thr853, and it facilitated Thr853 phosphorylation. Also, Khasnis et al., (2014) showed that the Thr696(s) of endogenous MYPT1 are spontaneously phosphorylated in quiescent human leiomyosarcoma cells. They suggested that the spontaneous phosphorylation at Thr696 might mediate the basal activity of myosin light chain phosphatase and the temporal phosphorylation at Thr853. The spontaneous phosphorylation at Thr696 synchronised with myosin targeting. So, phosphorylation at Thr696 seems to be more important and initiate Ca^{2+} sensitization. Moreover, protein kinase C (PKC)-mediated phosphorylation of CPI-17 (PKC-potentiated inhibitory protein for protein phosphatase-1 of 17 kDa) at Thr38 also ensures myosin light chain phosphatase inhibition (Kitazawa et al., 2003).

Apart from PKC and RhoA/Rho kinase, receptor-mediated tyrosine kinase activation has also been implicated in Ca^{2+} sensitization via activation of the RhoA/Rho kinase pathway in rat aortic rings (Seok et al., 2008). Using human prostate tissues, Kunit et al., (2014) showed that two different focal adhesion kinase inhibitors, PF-573228 and Y-11, inhibited α_1-AR-mediated smooth muscle contraction. Their data suggested that focal adhesion kinase was activated by α_1-ARs and was involved in smooth muscle contraction in the hyperplastic human prostate. Pharmacological inhibition of focal adhesion kinase impaired contraction in isolated human bladder smooth muscle cells and prostate, suggesting similar functions of focal adhesion kinase in the two tissues (Kunit et al., 2014; Luo et al., 2013).

In contrast, increased myosin light chain phosphatase activity by reduced Thr855 phosphorylation of MYPT1 or reduced Thr38 CPI-17 phosphorylation shifts the $[Ca^{2+}]i$: force ratio, effectively reducing Ca^{2+} sensitivity (Da Silva-Santos et al., 2009). Smooth muscle relaxation results from a reduction in Ca^{2+}-sensitization of myosin light chain phosphorylation and force development. For example, NO triggers vascular smooth muscle relaxation by stimulating the cyclic GMP formation and activating protein kinase G. Protein kinase G activation results in a desensitisation of myosin light chain phosphorylation to Ca^{2+} by phosphorylating MYPT1, resulting in myosin light chain phosphatase activation (Surks et al., 1999). Protein kinase G also mediates RhoA phosphorylation, that inhibits activation and membrane translocation (Kato et al., 2012); hence, mediating RhoA-mediated Rho kinase inhibition (Sauzeau et al., 2000).

1.1.2.5 Desensitisation of smooth muscle responses

G-protein-coupled receptors are under tight regulation which protects against the potentially harmful effects of acute or chronic overstimulation. As part of this regulation, α_1-ARs undergo regular turnover which involves internalisation and recycling back to the membrane (Morris et al., 2004; Stanasila et al., 2008). The constitutive internalisation of α_{1A}-AR involves clathrin-mediated endocytosis and the receptors are not degraded in lysosomes (Morris et al., 2004). Thus, the surface receptor density is maintained by recycling during constitutive internalisation (Morris et al., 2004).

The waning of GPCR signalling and loss of drug responsiveness in the continued presence of an agonist involves a co-ordinated series of events. These events include receptor uncoupling, sequestration, and down-regulation. Like many other GPCRs, α-ARs undergo agonist-promoted desensitisation, initiated by phosphorylation of the receptor in its third intracellular loop (Diviani et al., 1997). The well-characterized family of serine/threonine kinases termed GPCR kinases (GRKs) (Price et al., 2002), and second messenger-dependent kinases (Diviani et al., 1997) mediate phosphorylation of GPCR. For example, Diviani et al., (1997) showed that PKC mediates phosphorylation of Ser394 and Ser400, whereas GRK targets Ser404, Ser408, and Ser410 of the α_{1B}-AR.

GRK phosphorylation alone has little effect on G-protein coupling in the absence of arrestin. The role of GRK phosphorylation is to increase the affinity of the receptor for arrestin (Lohse et al., 1992). Binding of arrestin to the GPCR inhibits further receptor coupling to G-proteins. Furthermore, arrestin-bound GPCRs interact with clathrin and a β_2-adaptin subunit of the clathrin adaptor AP-2 (adaptor protein complex-2) to target the receptor to clathrin-coated pits and the endocytic machinery, leading to receptor internalisation (sequestration) (Naga Prasad et al., 2002) (Figure 1.8). Using fluorescence microscopy Laporte et al., (2000) reported that arrestin/ AP-2 interactions are essential for compartmentalisation and targeting of β_2-AR to clathrin for trafficking.

The AP-2 complex directly links the clathrin coat with transmembrane cargo proteins that sort into coated pits and vesicles. The AP-2 adaptor can initiate endocytosis of membrane receptors by either associating directly with the receptor cytoplasmic tail or by interacting

with additional molecules, such as arrestins, as described for the β_2-AR in COS-1 cells (Kim et al., 2002). Also, Conner et al., (1997) observed that arrestin knockout mice developed normally, but the normal adrenergic responses required arrestin. Depending on the GPCR subtype, arrestin either dissociates from the receptor at the plasma membrane or after co-internalization with the receptor.

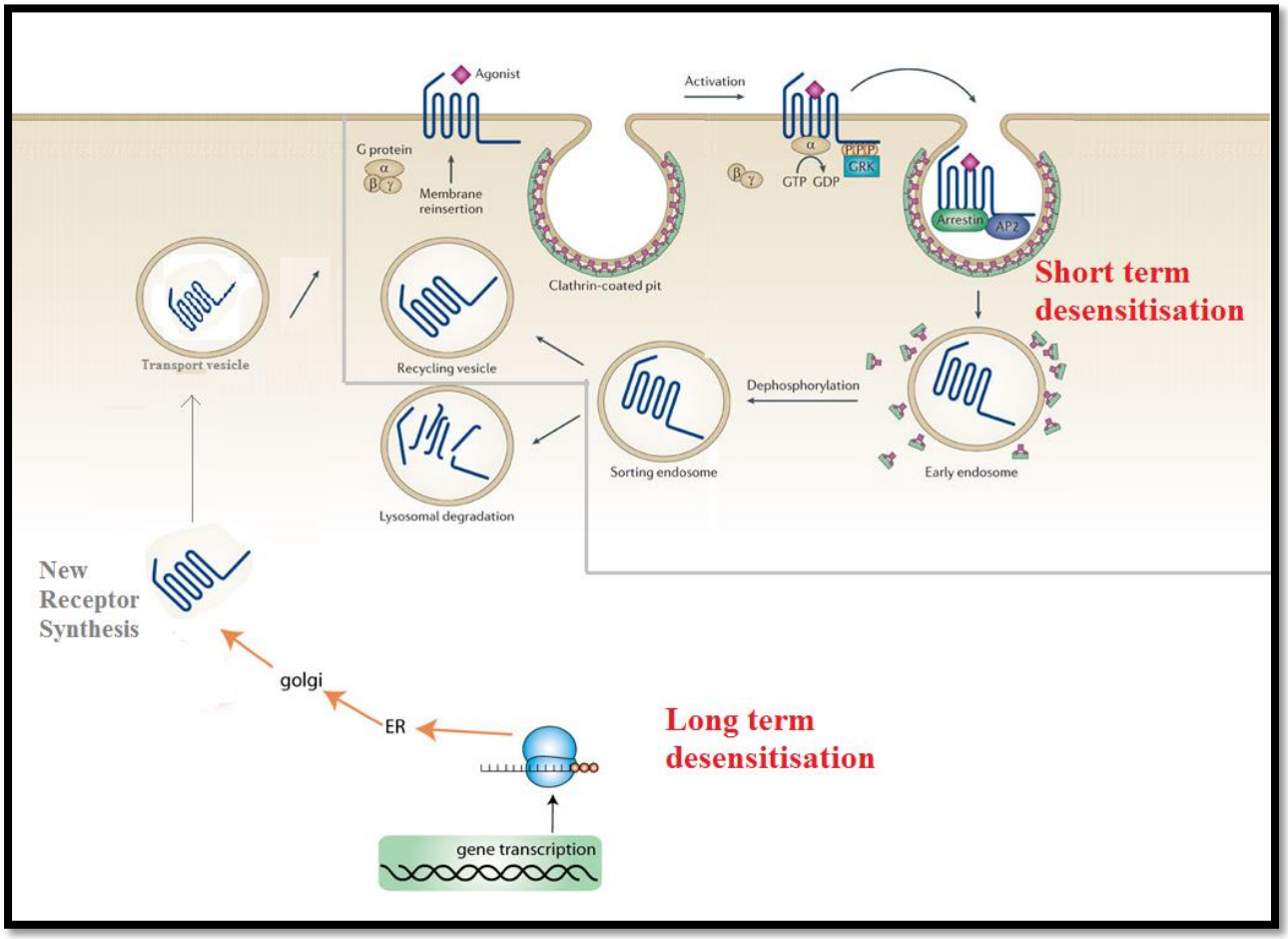


Figure 1.8 The mechanism of desensitisation, sequestration, re-sensitisation and downregulation of the G-protein-coupled receptor (GPCR). Desensitisation involves phosphorylation by a kinase, e.g., GPCR kinase (GRK), uncoupling of the G-proteins from the GPCR by the arrestin, transfer to the clathrin-coated pits and possibly endocytosis. From the early sorting endosomes, receptors separated from other adapters e.g. adaptor protein complex (AP-2) are moved to the recycling endosomes for membrane reinsertion and re-sensitisation (short-term desensitisation). On the other hand, long-term desensitisation and downregulation may involve lysosomal degradation of the receptors, gene transcription, and reinsertion of the receptor into the membrane. Modified from (Ramachandran et al., 2012). ER= endoplasmic reticulum.

Two other potent regulators of GRK activity are phosphatidylinositol 4, 5-bisphosphate and Gβγ, which are also critical for the membrane targeting of these kinases (Pitcher et al., 1995; DebBurman et al., 1996). Other important GPCR trafficking pathways include caveolae and lipid rafts (Xiang et al., 2002; Escriche et al., 2003).

In addition to kinases such as GRK, the regulation of GPCR is also modulated by molecules such as the mitogen-activated protein kinase (MAPK) (Keffel et al., 2000). These kinases phosphorylate many transcription factors and other kinases that are important regulators of gene expression (Heckler et al., 2014). The MAPK subgroups include the extracellular signal-regulated kinase, c-Jun *N*-terminal kinase, and p38 MAPK. The GRKs and arrestins activated during α_1-AR signalling activate the GPCR-mediated MAPK signalling cascade, e.g., for β-AR (Daaka et al., 1998; Copik et al., 2015) and α_{1A}-AR (Perez-Aso et al., 2013). Thus the MAPK signalling pathway also regulates the GPCR activities.

Internalisation of GPCRs, also termed receptor sequestration or endocytosis, occurs more slowly than desensitisation, happening over a period of several minutes after agonist exposure (Figure 1.8). The process of GPCR sequestration is important not only in attenuating GPCR signalling in the continued presence of agonist but also for receptor re-sensitisation and downregulation (Sibley et al., 1986). The re-sensitisation of a GPCR requires the dephosphorylation and dissociation of the receptor from its ligands.

Receptor down-regulation, defined as the loss of receptors from the cell membrane, is another critical component of receptor regulation. Downregulation of GPCRs is the persistent loss of cell surface receptors that occurs over a period of hours to days. When stimulation is chronically persistent for hours and days, internalised receptors may be degraded in the lysosomes (Figure 1.8) (Moore et al., 1999).

Short term and long term desensitisation

Receptor internalisation and degradation and/or recycling may influence the rate of recovery of responses to the ligand in question. For most GPCRs, internalisation following relatively short-term agonist treatment (within seconds to minutes) leads to dephosphorylation and recycling of the GPCR. The dissociated receptor may be inserted into the plasma membrane (re-sensitisation) (short-term desensitisation). Goral et al., (2011) observed that in A431 cells

expressing β_2-ARs, the shorter the initial stimulation, the greater the re-sensitization of the β_2-AR.

On the other hand, long-term desensitisation includes downregulation of surface receptor number, which involves internalisation, and degradation of receptors (Carter & Hill, 2005). In addition to the role of GRK in regulation of short-term desensitisation, GRK-mediated phosphorylation is an important mechanism underlying long-term desensitisation of the β_2-ARs in COS-7 cells (McGraw et al., 1998). Long-term desensitisation is associated with loss of surface receptors as shown for human lung mast cell β-AR (Chong et al., 2003; Scola et al., 2004). For example, prolonged exposure to isoprenaline decreased the net number of expressed β_2-AR (Nantel et al., 1995) and reduced the extent to which isoprenaline inhibited histamine release (Bastan et al., 2014). Also, studies in a human epithelial cell line indicated that long-term treatment with β-AR agonists led to reductions in receptor density, and the degree of internalisation is proportional to the agonist concentration (Williams et al., 2000). Long-term desensitisation could also lead to receptor downregulation, for example, Yin et al., (2016) showed that isoprenaline induced downregulation of β-ARs in the rat heart by an increase of mRNA degradation and not inhibition of transcription. Moreover, the human Antigen-R, the RNA binding protein, which promotes RNA degradation, was responsible for downregulation of β-ARs (Yin et al., 2016). Human Antigen-R recognises reiterated AUUUA sequences and was implicated in the regulation of RNA stability of β_1-AR and β_2-AR through binding to their untranslated regions (Bai et al., 2006; Kandasamy et al., 2005).

Inhibition of DNA transcription has also been proposed to be responsible for β-AR downregulation. However, the expression and activation of cyclic AMP-response element binding protein (CREB) is not changed during downregulation and thus, excluded CREB from receptor downregulation. CREB is a 43-kD basic leucine zipper transcription factor which regulates the transcription of many genes by binding to the CRE, an 8-bp palindromic consensus element (TGACGTCA), in their promoters (Maureira et al., 2016). CREB regulates the transcription of both α_1-AR (Thonberg et al., 2002), and β-AR (Lee et al., 2013; Sato et al., 2011).

Homologous desensitisation

With homologous desensitisation, only responsiveness to the receptor that is activated is lost. GRK-mediated phosphorylation of GPCR initiates homologous desensitisation, resulting in increased affinity of the phosphorylated receptor for arrestin (Diviani et al., 1996). GRK-mediated phosphorylation of serine or threonine residues in the third intracellular loop or COOH-terminus of the GPCR contribute to the arrestin binding (Seibold et al., 2000). GRK2 and GRK3 were shown to be involved in phosphorylation of agonist-occupied α_{1B}-ARs during homologous desensitisation (Diviani et al., 1997). GRK-mediated GPCR phosphorylation and binding of arrestin to the receptor facilitates agonist-promoted endocytosis of many GPCRs, including the β_2-ARs and M_2-M_5 mAChRs (Lee et al., 1998; Nobles et al., 2011).

Heterologous desensitisation

Phosphorylation of serine and threonine residues within the cytoplasmic loops and C-terminal tail domains of many GPCRs by protein kinases, including protein kinase A and PKC, induces heterologous desensitisation. Heterologous desensitisation does not require agonist occupancy of the target GPCR. Thus, activation of second-messenger-dependent protein kinases mediates desensitisation of the receptors that have not bound agonist, including receptors for other ligands (Molina-Muñoz et al., 2008; Rodríguez-Pérez et al., 2009). For example, α_1-ARs activation desensitises thromboxane prostanoid (TP) receptors through activation of PKC in the aorta of adult spontaneously hypertensive rats (Zhao et al., 2015).

Also, activation of lysophosphatidic acid receptor, sphingosine 1-phosphate receptor and oestrogen receptor induces α_{1B}-AR phosphorylation (Casas-González et al., 2000; 2003; Castillo-Badillo et al., 2012; Garcia-Sainz et al., 2000; González-Arenas et al., 2006). The phosphorylation, desensitisation and internalisation were via a signalling process involving phosphoinositide-3-kinase (PI3K) and PKC (Castillo-Badillo et al., 2012). Moreover, activation of receptors coupled to $G_{q/11}$, such as endothelin ET_A (Vázquez-Prado et al., 1997) or bradykinin BK2 (Medina et al., 1998) receptors , or receptors with intrinsic tyrosine kinase activity (such as epidermal growth factor), or platelet-derived growth factor receptors (Del

Carmen Medina et al., 2000) can induce α_{1B}-AR phosphorylation/desensitization. Thus PKC mediates heterologous desensitisation of GPCR.

Protein Kinase C (PKC) and desensitisation

Agents which activate PKC, such as GPCR agonists and phorbol esters, attenuate responses to AR agonists (Grandordy et al., 1994). Protein kinase C activation enhances homologous desensitisation of β_2-ARs (Boterman et al., 2006). Likewise, PKC-mediated desensitisation of α_{2A}-ARs function is primarily due to phosphorylation of serine 360 (Liang et al., 2002). Nishizaki & Sumikawa, (1998) demonstrated that PKC or protein kinase A phosphorylation of nicotinic acetylcholine receptors accelerated the rate of desensitisation.

There is a marked cross-talk between protein kinases, for example, Winstel et al., (1996) showed that the PKC phosphorylates, and membrane targets GRKs in CHO cells. Protein kinase C can mediate changes in the cellular expression and activity of GRKs (De Blasi et al., 1995); can mediate phosphorylation of GRKs (Chuang et al., 1995) and can target GRKs to the plasma membrane (Winstel et al., 1996). PKC phosphorylates GRK, and this phosphorylation relieves the inhibitory effect exerted by calmodulin on GRK. However, PKC phosphorylation can also be inhibitory on GRK (Pronin et al., 1998). Thus, PKC can modulate desensitisation or serve as a feedback loop for GRK homologous desensitisation mechanisms.

Rho kinase and desensitisation

RhoA-GTP, which activates Rho kinase, is a binding partner of GRK (Robinson & Pitcher, 2013). Also, arrestin expression and interactions were upregulated in mesenteric arteries in patients and rats with cirrhosis, resulting in GPCR desensitisation, G-protein dysfunction and Rho kinase inhibition (Chen et al., 2013). This suggests a relationship between arrestin and Rho kinase. Arrestins have also been shown to mediate β_2-AR signalling to the RhoA (Ma et al., 2012) and MAPK pathways (Gao et al., 2004). Arrestin forms a complex with Rho guanine nucleotide exchange factor in the cytosol in resting RCC7 or HEK-293 cells (Ma et al., 2012). Upon β_2-AR activation, both arrestin and Rho guanine nucleotide exchange factor translocate to the plasma membrane, with concomitant activation of RhoA. This suggested that arrestin may serve as a convergence point for non-$G_{12/13}$ and non-G_q protein-coupled

receptors to activate RhoA. All these observations suggest that Rho kinase can modulate desensitisation.

Calcium and desensitisation

Cellular Ca^{2+} levels appear to modulate GRK activity, Ca^{2+}-sensing proteins interacting with different GRKs (Sallese et al., 2000). Calmodulin, a universal mediator of Ca^{2+} signals, inhibited GRK binding to the plasma membrane (Sallese et al., 2000), suggesting that calmodulin may serve a general role in mediating Ca^{2+}-dependent regulation of GRK activity (Pronin et al., 1997). In HEK-293 cells expressing the $P2Y_{11}$ receptor, Haas et al., (2014) detected a significant reduction in the intracellular Ca^{2+} level in the course of 30-minutes ATP treatment. After a 60-minutes recovery period, the cells showed only a slightly recovered Ca^{2+} response probably because of the internalisation of the $P2Y_{11}$ receptors. This long-term desensitisation reflects a decrease in $[Ca^{2+}]_i$ during desensitisation.

Beta-ARs desensitisation

Most studies on desensitisation of AR have been performed on β-AR. An intense stimulation of the β_2-AR by β-AR agonists often leads to rapid desensitisation due to uncoupling of the receptor from Gs protein and adenylate cyclase. Prolonged activation leads to internalisation of the receptor into an intracellular endosome, thereby effectively removing the receptor from the cell surface. Desensitisation may lead to degradation and permanent receptor downregulation defined by an overall decrease in receptor number and the corresponding intracellular cyclic AMP accumulation (Oehme et al., 2015).

Regulation of β-AR includes two modes of desensitisation, one mediated by protein kinase A and another by GRK as shown in HEK-293 cells (Tran et al., 2004). Protein kinase A phosphorylation of β-ARs can lead to a "switch" in β-AR coupling from the Gs to Gi subtype of GTP-binding proteins (Zamah et al., 2002). Activated Gi proteins can then release GiαGTP and Giβγ subunits that can directly inhibit the catalytic activity of adenylate cyclase, thus reducing cyclic AMP production.

Incubation of human airway smooth muscle cells with salmeterol short-term (10 minutes) or long-term (2 and 14 hours) depresses isoprenaline stimulation (Gimenez et al., 2015).

Isoprenaline and salbutamol induces a significant concentration- and time-dependent decrease in β_2-ARs density (Oehme et al., 2015). Moreover, both isoprenaline and salbutamol reduce cyclic AMP generation in a concentration-dependent manner. Upon repeat exposure, all β_2-AR agonists produce functional desensitisation of β_2-ARs to the same degree and rate (Rosethorne et al., 2015). Moreover, the β_2-AR agonists are capable of causing robust receptor internalisation and arrestin recruitment, the rate of which is influenced by agonist efficacy. Pre-treatment with isoprenaline decreases isoprenaline-induced cyclic AMP generation compared with isoprenaline for 10 minutes alone, paralleled by increases in β_2-AR and GRK2 phosphorylation (Albano et al., 2015).

Isoprenaline (1μM) stimulation of β_2-ARs leads to rapid desensitisation and trafficking of the receptors to lysosomes (Macia et al., 2012: Samoshkin et al., 2015). Moreover, previous reports showed that Rab5 protein orchestrates internalisation and endocytotic trafficking of β-ARs in the endothelial cells (Yang et al., 2015) and human airway smooth muscle cells (Jiang et al., 2012).

Also, Rumzhum et al., (2016) recently showed that β_2-ARs can undergo heterologous desensitisation induced by bioactive sphingolipid acting on the sphingosine 1-phosphate receptor. The treatment of the airway smooth muscle cells with sphingosine 1-phosphate (1μM) for up to 24 hours induced heterologous β_2-ARs desensitisation. Thus desensitisation of β-AR is mediated by either protein kinase A or GRK as shown in airway smooth muscle.

1.1.2.6 Heterologous G-protein coupled receptor sensitisation

Heterologous-induced GPCR-mediated sensitization is a known phenomenon, at least in neuronal and other cell cultures of the airways smooth muscles. For example, Magalhaes et al. (2010) have demonstrated that the activation of the corticotropin-releasing factor receptor-1 resulted in the 'heterologous sensitization' of 5-HT_2R signalling in both heterologous cell cultures and primary prefrontal cortical neurones. Specifically, pre-stimulation of the corticotropin-releasing factor receptor-1 led to a significant potentiation of 5-HT_2R signal transduction and enhanced 5-HT_2R receptor-mediated inositol phosphate formation. Also, in the HEK-293 cells, Kurian et al., (2009) showed that $G\alpha q/_{11}$-coupled M_3 -mAChR facilitated Ca^{2+} signalling by Gαs-coupled β_2-AR. The crosstalk required Gαs and was dependent upon

intracellular Ca^{2+} release channels, particularly inositol 1, 4, 5-tris-phosphate receptors (Kurian et al., 2009). Therefore, receptor sensitization seems to be associated with Ca^{2+} mobilisation.

1.1.2.7 Urothelium function in the lower urinary tract

The urothelium serves as a transducer; communicating physical and mechanical stimuli to the underlying cells via secretion of chemical mediators and activities of expressed receptors. Little is known about the receptors of the urethral urothelium but that of the bladder is well studied and the literature is summarised below.

Muscarinic receptors (mAChRs) of the urothelium

Messenger RNA for all five mAChR subtypes have been shown in the rat (Kim et al., 2008) and human (Mansfield et al., 2005; Tyagi et al., 2006) bladder urothelium, and the expression of all five mAChR (M_1 - M_5) subtypes have been shown in the bladder urothelium of mouse (Zarghooni et al., 2007) and human (Bschleipfer et al., 2007). The urothelial cells express a high density of mAChRs, even greater than the bladder smooth muscle cells, as shown in the porcine urinary bladder (Hawthorn et al., 2000). Data from cultured rat urothelial cells, has suggested that activation of M_{1-3} mAChR subtypes stimulates the release of ATP through an increase of intracellular Ca^{2+} (Kullmann et al., 2008b; Sui et al., 2014), which might modulate bladder responses.

Nicotinic receptors of the urothelium

Beckel et al., (2006) identified mRNA for the α_3, α_5, α_7, β_3, and β_4 nicotinic subunits in rat urothelial cells using real time-PCR, and the expression of nicotinic acetylcholine receptors in the urothelium was shown in the rat (Beckel et al., 2006; Beckel & Birder, 2012) and mouse bladder (Zarghooni et al., 2007). Stimulation of nicotinic acetylcholine receptors via the intravesical administration of nicotine increased the inter-contraction interval of rat bladder (Beckel et al., 2006). The specific activation of α_7 homomeric receptors inhibited bladder reflexes while α_3 heteromeric receptors had an excitatory effect on bladder reflexes (Beckel et al., 2006). Stimulation of the α_7 receptors and the α_3 heteromeric receptors with low concentrations of acetylcholine inhibited the release of urothelial ATP (Beckel & Birder,

2012). However, stimulation with high concentrations of acetylcholine caused the α_3 heteromeric receptors to stimulate the ATP release. Thus, the nicotinic acetylcholine receptor appears to mediate bladder activity and reflexes by modulating urothelial release.

Alpha-adrenoceptors (α-ARs) of the urothelium

High levels of α_{1A}-AR mRNA were detected in the urothelium of the human bladder dome and bladder base (Walden et al., 1997). The α_{1A}-AR subtype is also expressed in the bladder urothelium of pig (Moro et al., 2013), rat (Yanase et al., 2008; Walden et al., 1997) and monkey (Walden et al., 1997). However, the α_{1A}-ARs were undetectable at the protein level in the human bladder (Walden et al., 1997). Thus, the α_{1A}-AR mRNA appeared to be transcribed, but not translated in the human urothelium. However, in the pig, $\alpha_{1/L}$-ARs mediate an increase in the urothelium contractile rate and tension to phenylephrine (Moro et al., 2013).

Similarly, the expression of α_{1D}-ARs was detected in rat urothelial tissue via western blot and immunohistochemistry (Ishihama et al., 2006), and endogenous catecholamines act on α_{1D}-ARs in the urothelium to facilitate mechanosensitive bladder afferent nerve activity and reflex voiding (Ishihama et al., 2006). Moreover, Kurizaki et al., (2011) found an increased α_{1D}-ARs mRNA expression in the bladder urothelium of men with lower urinary tract symptoms/benign prostatic obstruction. Men with a first desire to void (FDV) $\leq$ 200 ml and strong desire to void (SDV) $\leq$ 300 ml expressed more α_{1D}-AR mRNA compared with patients having FDV $\geq$ 201 ml and SDV $\geq$ 301 ml (Kurizaki et al., 2011). This suggests a role for urothelial α_{1D}-ARs in bladder sensation.

Beta-adrenoceptors (β-ARs) of the urothelium

The human (Otsuka et al., 2008; Tyagi et al., 2009) and pig (Moro et al., 2013) bladder urothelium express mRNA for β_1-, β_2- and β_3-ARs. Moreover, studies have shown the expression of β-ARs in the bladder urothelium of rat (Kullmann et al. 2009, 2011) and pig (Moro et al., 2013) and human (Limberg et al., 2010). Immunostaining for β_1, β_2, and β_3 was prominent in the female bladder urothelium (Limberg et al., 2010). Using radioligand techniques, Rojanathammanee et al., (2009) reported an overwhelming presence of β_2-ARs in UROtsa cells, and their activation protected the cells against programmed cell death.

Beta-AR stimulation activates the adenylate cyclase pathway (Limberg et al., 2010) in bladder urothelial cells and initiates an increase in intracellular Ca^{2+} that triggers NO production and release (Birder et al., 2002b). Moreover, Moro et al., (2013) showed the β_2-AR subtype induced inhibition of porcine urothelium spontaneous contractile activity. The β_1-ARs and β_2-ARs were possibly involved in the relaxation of the tension responses (Moro et al., 2013).

Purinergic receptors of the urothelium

There is substantial evidence that many of the purinergic receptor subunits are expressed throughout the urinary bladder urothelium of animals (Birder et al., 2004; Chopra et al., 2008; Kim et al., 2008) and human (Shabir et al., 2013). In the human bladder urothelium, glycosylated $P2X_2$ and $P2X_3$ transcripts and protein expression were detected (Tempest et al., 2004). Furthermore, in the human urothelial cell line, UROtsa, $P2Y_1$, $P2Y_2$, and $P2Y_{11}$ transcript expression were detected (Save & Persson, 2010). Wang et al., (2005) showed that upon binding of urothelially released ATP to P2Y and P2X receptors on the umbrella cell, downstream Ca^{2+} and protein kinase A may act to stimulate membrane insertion at the apical pole of umbrella cells.

Prostanoid receptors of the urothelium

There are nine prostanoid receptors classified as DP_1, DP_2 ($CRTH_2$), EP_1, EP_2, EP_3, EP_4, FP, IP and TP, which are preferentially activated by the major prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$ PGI_2 and TxA_2. Immunohistochemical analyses revealed that EP_1 and EP_2 receptors are expressed in the guinea-pig bladder urothelium (Rahnama'i et al., 2010). Moreover, the expression of the prostaglandin E receptors, EP_1, EP_2, EP_3, and EP_4, and mRNA for EP_2, EP_3, and EP_4 were reported in dog urethral urothelium (Ponglowhapan et al., 2010). Thus, locally released prostanoid by the smooth muscle or the urothelium may act in an autocrine or paracrine manner to modulate the activity of the bladder and urethra via the urothelium.

1.1.2.8 Urothelium released factors and function of the lower urinary tract

The urothelium secretes factors including ATP, acetylcholine, NO (Apodaca et al. 2007) and nerve growth factor (NGF) (Birder et al., 2007) through the apical and/or serosal surfaces in response to mechanical, chemical, and thermal stimuli (Birder & Andersson, 2013). These factors diffuse to underlying submucosal sensory nerve fibres and ICCs and appear to be involved in normal bladder function (Birder et al., 2002a).

Urothelial released ATP

The urothelium is capable of releasing ATP into both the mucosal and serosal compartments of the bladder wall (Ferguson et al., 1997; Lewis & Lewis et al., 2006) and ATP act as an important autocrine (Wang et al., 2005) and paracrine mediator (Cockayne et al., 2000), respectively. The paracrine action of ATP released from urothelial cells induces detrusor smooth muscle contraction in the guinea-pig and human (Sui et al., 2014). Urothelial ATP was released mainly via vesicular transport or exocytosis in rabbit bladder (Wang et al., 2005) or pannexin-1 hemichannels conductive efflux in mice (Negoro et al., 2013) and rat bladder (Beckel et al., 2015; Timóteo et al., 2014). Timoteo et al., (2014) reported co-localisation of pannexin-1 hemichannels and $P2Y_6$ receptors in the rat urothelium. Other mechanisms implicated in ATP release by the urothelial cells include (i) uridine triphosphate- (UTP-) mediated Ca^{2+}-dependent ATP release and (ii) agonist-mediated Ca^{2+}-independent ATP release (Sui et al., 2014).

ATP released from both the apical and basolateral urothelial surfaces acts via P2X and possibly P2Y receptors on the umbrella cells to stimulate stretch-induced exocytosis, as well as endocytosis in the rabbit (Wang et al., 2005). Moreover, ATP released from the basolateral surface of the bladder urothelium of mice during bladder filling stimulates $P2X_3$ receptors on suburothelial sensory nerve fibres, thus relaying information about the degree of bladder filling to the central nervous system (Cockayne et al., 2000; 2005).

Increased ATP released from the urothelium may, in part, underlie the development of lower urinary tract symptoms in bladder dysfunction (Munoz et al., 2011). The dysfunction is

thought to be due to increased firing rate of bladder afferent nerves inducing bladder overactivity (Pandita & Andersson, 2002; Yu & de Groat, 2008).

Urothelial released acetylcholine

Parasympathetic nerves are not the only source of acetylcholine, as it can also be formed and released non-neuronally by the urothelium (Hanna-Mitchell et al., 2007; Lips et al., 2007; McLatchie et al., 2015; Yoshida et al., 2006). The urothelium contains the essential components of the machinery needed to synthesise acetylcholine including carnitine acetyltransferase, but the presence of choline acetyltransferase remains controversial (Hanna-Mitchell et al., 2007; Lips et al., 2007). Hanna-Mitchell et al., (2007) reported that rat bladder urothelial cells expressed choline acetyltransferase. However, RT-PCR data from human and mouse urothelial cells by Lips et al., (2007) does not support the expression of choline acetyltransferase. The variation in result may be associated with species differences. The urothelium lacks the vesicular acetylcholine transporter, which is characteristic for cholinergic neurones, but rather expresses the polyspecific organic cation transporters OCT1 and OCT3 (Lips et al., 2007). In the urothelium, acetylcholine may act on nicotinic acetylcholine receptors as well as on mAChRs (Beckel & Birder, 2012; Zarghooni et al., 2007).

Unlike neuronal acetylcholine released from parasympathetic nerves, which occurs largely during the voiding phase, urothelial acetylcholine release is promoted during bladder filling, that is, by stretch (Yoshida et al., 2006; 2008). Therefore, urothelial acetylcholine release is likely to occur during the storage phase.

Urothelial released nitric oxide (NO)

Formation of NO can occur in urothelial cells via inducible nitric oxide synthase (iNOS) (Chuang et al., 2013) or endothelial nitric oxide synthase (eNOS) (Birder et al., 2002b). Some reports also implicate nNOS in urothelial release of NO (Gillespie et al., 2005). Nitric oxide can be released from urothelial cells upon mechanical force or stretch, mimicking bladder distension (Birder et al., 2002a). Moreover, NO is released from the urothelium upon activation of mAChRs (Andersson et al., 2012; Kullmann et al., 2008a), vanilloid receptors

(Birder et al., 2001) and ARs (Birder et al., 2002b). The urethral urothelium releases NO in response to electrical field stimulation in the hamster (Pinna et al., 1999).

Urothelial released prostanoids

Prostanoids are produced locally within the bladder urothelium in the human and other species (Kang et al., 2015a; Jeremy et al., 1987). The umbrella cells of guinea-pig bladders do not express cyclooxygenase I, however, cyclooxygenase I was present in basal urothelial cells, making them a possible site of prostaglandin synthesis (Rahnama'i et al., 2010). Guan et al., (2014b) showed that prostaglandin E_2 and prostaglandin D_2 were released mainly from urothelium of guinea-pig urinary bladders. Moreover, reports have shown that prostaglandin E_2 is synthesised in the rabbit (Klausner et al., 2011) and rat (Masunaga et al., 2006) bladder urothelium. Stretch of the rabbit bladder also mediates urothelial prostanoid production (Downie & Karmazyn, 1984).

Prostaglandin E_2 contributed to the detrusor spontaneous rhythmic activity in the mouse (Klausner et al., 2011; Kobayter et al., 2012). Moreover, prostaglandin D_2 inhibited the motility of guinea pig urinary bladder induced by nerve stimulation, and post-junctional action of acetylcholine or ATP (Guan et al., 2014b). Prostaglandin E_2 release from the guinea pig bladder urothelium was modulated by the complex interaction between ATP, NO (Nile et al., 2010), acetylcholine and M_2 mAChRs (Nile & Gillespie, 2012). Nile & Gillespie, (2012) showed that activation of M_2 receptors induced production of prostaglandin E_2, while in the presence of NO donor, prostaglandin E_2 production was inhibited.

1.1.2.9 Interstitial cells and spontaneous contractile activity in the lower urinary tract

The lower urinary tract and urogenital tract develops spontaneous contractile activity. Such spontaneous contractile activity was initially considered to originate from the smooth muscle cells themselves, and thus often referred to as ‘myogenic activity.' However, with the establishment of the ICC as the primary pacemakers, driving slow wave generation in the gastrointestinal tract, many regions of the urogenital tract have been examined for the presence of similar cells. Interstitial cells of Cajal-like cells have been identified in many

regions of the lower urinary tract by immunohistochemical studies and electron microscopy (Davidson & McCloskey, 2005; Johnston et al., 2010; Kubota et al., 2008).

In the bladder, myofibroblasts in the lamina propria have a morphology similar to ICC-LCs and can also generate spontaneous electrical and Ca^{2+} activity (Wu et al., 2004). Interstitial cells of Cajal-like cells in the bladder are preferentially located along the boundary of smooth muscle cell bundles where many spontaneous smooth muscle cell Ca^{2+} transients originate (Hashitani et al., 2001; McCloskey & Gurney, 2002).

Sergeant et al., (2000) have shown that the rabbit urethra possesses ICCs, with structural and morphological properties similar to those found in the gastrointestinal ICC. Urethral ICC-LCs in situ and after enzymatic isolation generated spontaneous Ca^{2+} transients relying on Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} influx through non-L-type Ca^{2+} channel pathways (Hashitani & Suzuki, 2007). Urethral ICC-LCs Ca^{2+} transients are consistently recorded at lower frequencies and have a longer duration than the Ca^{2+} transients recorded in neighbouring smooth muscle bundles of guinea pig bladder (Hashitani et al., 2004a) and mouse' renal pelvis (Lang et al., 2007a;b). Interstitial cells of Cajal isolated from the urethra generated slow waves through a combination of Ca^{2+} release from the internal stores and the opening of Ca^{2+}-activated Cl^{-} channels (Hollywood et al., 2003b). Spontaneous Ca^{2+} transients observed in ICC-LCs of the rabbit urethra in-situ occurred at a frequency of 1–10/min. The Ca^{2+} transients also have a much longer duration (5–30 sec.) than the Ca^{2+} transients in the urethral smooth muscle cells (1–3 sec. duration) measured in same preparation (Hashitani & Suzuki, 2007). Thus the lower urinary tract expresses ICC-LC with pacemaker activity.

1.1.2.10 Urine storage and micturition

Normal coordination of storage and voiding functions requires integration via the pontine micturition centre and the pontine storage centre and has supraspinal input (Blaivas, 1982; Thor et al., 1990). The switch of lower urinary tract function between storage and voiding is mediated by a long-loop spino-bulbospinal voiding reflex which has its rostral terminus in the brainstem (De Groat et al., 2015; Noto et al., 1991). The pontine micturition centre coordinates brain centres including the periaqueductal gray. The periaqueductal gray is a key

organising centre for several higher brain regions involved in micturition, including the prefrontal cortex with which it has strong connections (Sugaya et al., 1997; Vizzard et al., 1995b).

Urine storage

During filling, there is low-level activity from bladder afferent fibres that signal via the pelvic nerve and this, in turn, stimulates sympathetic outflow to the bladder neck and wall through the hypogastric nerve. These fibres regulate relaxation of the detrusor via β_3-ARs and constriction of the urethra via α_1-ARs in the internal urethral sphincter. There is also pudendal outflow, which keeps the external urethral sphincter closed. Acetylcholine acts on nicotinic acetylcholine receptor in the striated muscle, inducing muscle contraction to maintain closure of the external urethral sphincter (Thor et al., 1989; Blaivas, 1982). Motor neurones innervating the striated muscles of the external urethral sphincter exhibit a tonic discharge that increases during bladder filling (Thor and de Groat, 2010). During accommodation, neurones within the pontine storage centre in the brain are quiescent (Michels et al., 2015; Tai et al., 2009).

The urethral outlet remains closed, and the external urethral sphincter contracts with greater and greater frequency while the bladder is filling. This progressive increase in activity of the external urethral sphincter during bladder expansion is known as the guarding reflex. As the bladder fills, bladder sacral afferent signals increase in strength until they exceed a certain threshold in the brainstem, specifically the periaqueductal gray.

Micturition

The switch from the storage to the micturition phase is elicited by slowly adapting mechanoreceptors in the urinary bladder wall (Gonzalez et al., 2014). The spinobulbospinal pathway consisting of an ascending sensory limb that passes from the sacral spinal cord to circuitry in the rostral brainstem mediates the micturition reflex. The micturition reflex leads to activation of neurones in the pontine micturition centre that send excitatory signals back to the sacral spinal cord to complete the reflex circuit (Noto et al., 1991; Tai et al., 2009). A complex integration of afferent signals along with conscious perceptions of how full one's bladder is and an appreciation of the social environment of the moment, mediates voiding.

When the urinary bladder fills to approximately 200-300 ml, there is an increase in afferent firing from tension receptors in the bladder which produces firing in the sacral parasympathetic pathways and inhibition of sympathetic and somatic pathways (Sugaya et al., 2003; Tanaka et al., 2003). At a critical level of bladder distention, the afferent activity arising from mechanoreceptors in the bladder wall switches the pontine micturition centre "on" and enhances its activity. At this point, the accommodation centre shuts "off" and ascending afferent input passes through the periaqueductal gray relay centre before reaching the pontine micturition centre and elicits efferent outflow (De Groat & Yoshimura, 2009). The signal stimulates the spinal cord, which responds with parasympathetic activity that relaxes the smooth muscle of the internal urethral sphincter and contracts the detrusor muscle. Postganglionic axons that terminate in the detrusor layer release acetylcholine onto the smooth muscle fibres, with consequent contractions of the bladder in processes mediated by mAChRs in detrusor cells. ATP is co-released with acetylcholine from the parasympathetic nerves. The acetylcholine and ATP released activate the mAChRs and purinergic receptors respectively (Tsai et al., 2012). Moreover, urine does not flow until a voluntary nerve impulse from the pudendal nerve relaxes the striated muscle of the external urethral sphincter.

1.1.3 Disorders of the lower urinary tract

Lower urinary tract symptoms can be divided into three groups, namely, storage symptoms (e.g. urgency, frequency, urinary incontinence, overactive bladder), voiding symptoms (e.g. hesitancy, straining, low flow rate, increased residual volume) and post-micturition symptoms (a feeling of incomplete emptying following urination) (Alexandre et al., 2016).

1.1.3.1 Overactive bladder (OAB)

Detrusor overactivity is the urodynamic observation characterised by involuntary detrusor contractions during the filling phase that may be spontaneous or provoked (Haylen et al., 2010). In men, detrusor overactivity may cause overactive bladder. Overactive bladder syndrome is characterised by urinary urgency, usually accompanied by frequency and nocturia, with or without urgency urinary incontinence, in the absence of urinary tract infection or other obvious pathology (Alexandre et al., 2016; Haylen et al., 2010). Overactive bladder has been shown to affect up to 36% of adult women in Europe and the U.S.A. (Coyne

et al., 2009). Neurogenic overactive bladder results from neurological disorders such as multiple sclerosis, spinal cord injury, stroke, or neurodegenerative diseases, while if the aetiology is unclear it is regarded as idiopathic or non-neurogenic overactive bladder. Detrusor smooth muscle taken from idiopathic overactive bladder have been shown to exhibit aberrant spontaneous activity, suggesting that the increased excitability of detrusor smooth muscles during this pathological condition may be attributed to the altered properties of the smooth muscle cells themselves (Sui et al., 2009). Research has also implicated alterations at the level of the ICC (Johnston et al., 2008). In support, imatinib, an inhibitor of Kit receptor tyrosine kinase, was potent at inhibiting evoked and spontaneous contractions in overactive bladder than in normal bladder (Biers et al., 2006).

The medical management of overactive bladder has focused on antimuscarinic agents such as oxybutynin, tolterodine, fesoterodine, trospium, darifenacin and solifenacin (Table 1.1). Oxybutynin is selective for M_1 and M_3 (Nilvebrant et al., 1997), tolterodine is selective for M_2 and M_3, darifenacin and solifenacin are selective M_3 mAChR antagonist (Steers, 2006). Also, fesoterodine and trospium are the non-selective mAChR antagonist. These drugs bind competitively to mAChRs to dampen the amplitude of bladder contractions, improving bladder capacity and reducing involuntary detrusor contractions, urgency, and frequency of micturition. Antimuscarinics may also decrease bladder afferent activity by blocking urothelial mAChRs; thereby further improving overactive bladder symptoms (De Laet et al., 2006).

The drawbacks of antimuscarinic treatment include limited tolerability due to the adverse effects such as dry mouth, constipation, blurred vision and cognitive impairment, especially in the elderly (Wagg et al., 2014). Despite more selective newer antimuscarinics, the drugs have limited long-term benefit (Jayarajan & Radomski, 2013; Veenboer & Bosch, 2014). Further concerns regarding antimuscarinics have been brought to light as recent cohort studies have correlated an increased likelihood of Alzheimer's disease or dementia in those who are undergoing cumulative anticholinergic exposure (Gray et al., 2015).

Table 1.1 Lower urinary tract symptoms disorders; definition, aetiology, pharmacotherapy and associated side effects.

Symptoms	Definition	Aetiology	Pharmacotherapy/mode of action	Other treatments	Side effects	References
Overactive bladder (OAB) Syndrome	Urinary urgency, usually accompanied by frequency.	Idiopathic & neuropathic.	Antimuscarinic such as oxybutynin, tolterodine, fesoterodine, trospium, darifenacin, solifenacin. These drugs competitively block the muscarinic receptors on the detrusor muscle. They dampen the amplitude of bladder contractions or /and decrease bladder afferent activity.		Dry mouth, constipation, blurred vision, and cognitive impairment.	Jayarajan & Radomski, 2013; Wagg et al., 2014.
			$Beta_3$-AR agonists (mirabegron, ritobegron, and solabegron). These drugs mediate detrusor relaxation.		Hypertension.	Nitti et al 2013; 2014.
			Botulinum toxin A; inhibition of vesicular release of neurotransmitters and attenuation of central sensitisation.		A headache, muscle stiffness, neck or back pain.	Hsieh et al., 2016; Wyndaele &Van Dromme, 2002.
Detrusor overactivity (DO)	Urodynamic observation characterised by involuntary detrusor contractions during the filling phase.	Idiopathic & neuropathic.	Antimuscarinics; competitively blocking the muscarinic receptors on the detrusor muscle (Oxybutynin, Tolterodine).	Surgery, intravesical Botox.	Dry mouth, constipation, blurred vision, and cognitive impairment.	Demaagd & Davenport, 2012.
Partial urethral obstruction	Voiding symptoms (urgency, frequency, slowing of the urinary stream, straining to void and nocturia).	Benign prostate hypertrophy, carcinoma.	The α-antagonists, such as terazosin, doxazosin, tamsulosin, and alfuzosin.	Surgery.	Postural hypotension	Buzelin et al., 1997; Hill, 2015; Na et al., 1998.
Interstitial cystitis	Chronic inflammatory bladder disease characterised by urinary frequency, urgency, and bladder/pelvic pain Afferent pathways are sensitised.		Antimuscarinics such as oxybutynin, tolterodine to control OAB symptoms.		Dry mouth.	Wagg et al., 2014.
			Pentosan polysulfate sodium which adheres to the bladder; and restores epithelial function.		Anticoagulant and fibrinolytic effects.	Fiehn & Kim, 2014.
			Antibiotics.		Gastric distress.	

Three β_3-AR agonists, mirabegron, ritobegron, and solabegron, have been studied in detail regarding their efficacy in treating overactive bladder (Chapple et al., 2014a; Nitti et al 2013; 2014; Ohlstein et al., 2012).

Cystometric experiments in rats treated with mirabegron have demonstrated a reduction of resting intravesical pressure and contraction frequency without any effect on the amplitude of micturition contraction (Takasu et al., 2007). Based on these findings, sponsored trials which recruited over 10,500 patients with overactive bladder were conducted (Chapple et al., 2013a, 2013b, 2013c; Herschorn et al., 2013; Kuo et al., 2014; Yamaguchi et al., 2014). The evidence described suggested mirabegron not being a cure for overactive bladder, but as efficacious as most antimuscarinics including tolterodine.

Ritobegron and solabegron are in differing phases of research while mirabegron is now available for the treatment of overactive bladder in many countries. The US Food and Drug Administration (FDA) approved mirabegron in June 2012 and the National Institute for Health and Care Excellence (NICE) in the UK in June 2013. Mirabegron is also licensed for overactive bladder treatment in Japan, Europe and Canada.

Recently botulinum toxin A was suggested for treatment of overactive bladder (Hsieh et al., 2016). The mechanism of botulinum toxin A includes inhibition of vesicular release of neurotransmitters and the axonal expression of capsaicin and purinergic receptors in the lamina propria, as well as attenuation of central sensitization (Hsieh et al., 2016). Hsieh et al., (2016) reported that in a multiple randomised, placebo-controlled trials, botulinum toxin A was an effective treatment for patients with refractory idiopathic or neurogenic detrusor overactivity. The urinary incontinence episodes, maximum cystometric capacity, and maximum detrusor pressure were improved greater by botulinum toxin A compared to placebo. Moreover, the adverse effects of botulinum toxin A, such as urinary retention and urinary tract infection, were primarily localised to the lower urinary tract.

Urgency urinary incontinence is the complaint of involuntary leakage accompanied by, or immediately preceded by urgency. Urgency urinary incontinence is associated with a sudden and strong desire to void as a result of detrusor overactivity or increased sensation in the overactive bladder (Abrams et al., 2002). In men with benign prostatic hyperplasia, urgency

urinary incontinence co-exists with overactive bladder syndrome or occurs after benign prostatic hyperplasia surgery (Han et al., 2007). Urgency urinary incontinence commonly occurred in elderly patients with stroke, Parkinson disease, multiple sclerosis, and various spinal cord disorders and Alzheimer disease (Lee et al., 2014; Na et al., 2015).

1.1.3.2 Bladder outlet obstruction (BOO)

In ageing men, lower urinary tract symptoms have been attributed to bladder outlet obstruction as a result of benign prostatic enlargement resulting from the condition of benign prostatic hyperplasia (Abrams et al., 2013). Bladder outlet obstruction is also common in men as a result of carcinoma, or calculi which increase back pressure during micturition.

During the early stages of urethral restriction due to benign prostatic hyperplasia, the bladder undergoes compensatory hypertrophy to produce the greater contractile force required to force urine past the restriction and keep residual urine volumes low. The primary effects of urethral obstruction are found in the bladder and can manifest as hydronephrosis which can threaten kidney function (Harrison et al., 1983). If bladder outlet obstruction is left untreated, the bladder wall ultimately decompensates and becomes hyperactive with the loss of functional capacity.

Apart from the overactive bladder and bladder outlet obstruction, interstitial cystitis is also a disorder of the lower urinary tract. Interstitial cystitis is a chronic inflammatory bladder disease characterised by urinary frequency, urgency, and bladder/pelvic pain. Afferent pathways are sensitised in interstitial cystitis (Parsons et al., 1998; Theoharides et al., 1995). Moreover, the release of ATP and NO are increased with interstitial cystitis (Logadottir et al., 2004; Sun & Chai, 2006). Antibiotics and pentosan polysulfate have been suggested to relieve the bladder pain or discomfort associated with interstitial cystitis (Table 1.1). Antibiotics and pentosan polysulfate are only effective in relieving bladder pain in about 30%-60% of patients. Moreover, they mediate unfavourable side effects, including nausea, diarrhoea, gastric distress, and hair loss (Fiehn & Kim, 2014).

1.1.3.3 Stress urinary incontinence (SUI)

Stress urinary incontinence (SUI), characterised by involuntary loss of urine during exertion, sneezing and coughing is most common in women, although, it does occur in men following prostatectomy (Khandelwal & Kistler, 2013). Stress urinary incontinence is a common condition affecting nearly half of all women suffering from urinary incontinence (Medina et al., 2016). The estimated worldwide number of women aged ≥20 years projected to have SUI or mixed urinary incontinence in 2018 is 153.5 and 52.5 million, respectively (Irwin et al., 2011). A recent study by Mckenzie et al., (2016) showed that prevalence of SUI in women was 49.3% in Western Australia (mean age of 39 years).

Stress urinary incontinence is characterised by impaired closure mechanisms of the urethra as a result of a weak pelvic floor or poorly supported urethral sphincter (urethral hypermobility) and/or a damaged urethral sphincter system (intrinsic sphincter deficiency). The two conditions result in impaired urethral closure and involuntary loss of urine (Khandelwal & Kistler, 2013; Yoshimura & Miyazato, 2012). In SUI animal models, both urethral striated and smooth muscles are significantly reduced (Badra et al., 2013). However, SUI is linked to intrinsic sphincter deficiency more than urethral hypermobility. Several studies have shown incontinence could emanate from damage to the urethral sphincter (Chaliha et al., 1999). Furthermore, pelvic floor musculature and hormonal changes coupled with the shorter urethra in the female contribute to the prevalence of SUI in female (Kristiansson et al., 2001; Tunn et al., 2006). Menopause, pregnancy, obesity, constipation, diabetes mellitus, pelvic organ prolapse and vaginal parity further predispose women to SUI (Brown et al., 2006; Zhu et al., 2009).

Present treatments for SUI have been limited largely to continence surgery and various types of behavioural interventions (conservative method); including pelvic floor muscle training (e.g. Kegel exercises) (Table 1.2). Hormonal, minimal invasive surgeries and pharmacotherapy are also treatments for SUI (Ishiko et al., 2001; Zyczynski et al., 2015). Although pelvic floor muscle training can be effective, it requires motivation on the part of the patient for up to 20 weeks; moreover, the dropout rate is high (Ashworth & Hagan, 1993).

Table 1.2 Types of incontinence, aetiology, present pharmacotherapy & treatments and associated side effects.

Type of Incontinence	Aetiology/ pathophysiology	Symptoms	Treatments Conservative management/ Surgery	Pharmacotherapy	Side effects	References
Urge urinary incontinence	Overactive bladder and increased afferent nerve activity, neurologic disorders, Spinal cord injury.	Loss of urine accompanied or preceded by a strong desire to void; may be accompanied by frequency and nocturia.	Weight loss, fluid intake reduction, constipation management, bladder training, pelvic floor muscle exercises, electrical stimulation of the posterior tibial nerve, and neuromodulation (implanted sacral nerve stimulation).	Muscarinic cholinergic receptor antagonist such as darifenacin, fesoterodine, oxybutynin, solifenacin, tolterodine, trospium which blocks muscarinic cholinergic detrusor receptors to inhibit detrusor contraction.	Dry mouth & blurred vision.	Ercan et al., 2015.
				Prostaglandin synthesis inhibitors which block prostaglandin-induced detrusor contraction.	Clinical evidence is still lacking.	Kibar et al., 2011; Tanaka et al., 2010.
				Beta-AR agonist such as mirabegron which induces detrusor relaxation by activating the β-ARs.	Hypertension and nasopharyngitis.	Nitti et al., 2013; 2014.
Stress urinary incontinence (SUI)	Loss of tone of the bladder neck/urethral internal sphincter, increased urethral mobility, intrinsic sphincter dysfunction.	Loss of urine with physical exertion or increases in intra-abdominal pressure (e.g., sneezing, coughing, and laughing).	Weight loss, smoking cessation, fluid intake reduction, constipation management, pelvic floor muscle exercises, electrical stimulation, sling procedures, urethropexy, colposuspension (i.e., Burch and Marshal-Marchetti-Krantz procedures) and periurethral injections of bulking agents.	Alpha-AR agonist such as pseudoephedrine, phenylephrine, phenylpropanolamine, ephedrine which induces contraction of the urethral sphincter/bladder neck upon activation of α -ARs.	Increased blood pressure, sleep disturbances, headache.	Segev et al., 2015.
				Beta-AR agonist such as mirabegron, ritobegron, and solabegron): induces detrusor relaxation.	Hypertension and nasopharyngitis.	Nitti et al., 2013; 2014.
				Noradrenaline and serotonin reuptake inhibitor (duloxetine) which induces contraction of the urethral sphincter/bladder neck upon activation of α-ARs and serotonin receptors.	Tiredness, dry mouth, nausea and constipation.	Alan et al., 2015.
				Oestrogen hormonal treatment: The oestrogen acts on the supporting tissue of the pelvic floor as well as increasing α-AR density, sensitivity, neuronal sensitivity, and metabolism.	Endometrial and breast cancer.	Cody et al., 2012.
Mixed urinary incontinence	Mix aetiology.	Combination of urge and stress symptoms.	Treatment depends on predominant symptoms.	Duloxetine which induces contraction of the urethral sphincter/bladder neck upon activation of α-ARs.	Nausea.	Alan et al., 2015.
				Antimuscarinic which blocks muscarinic cholinergic receptors in the detrusor to block detrusor contraction.	Dry mouth	Ercan et al., 2015.

Likewise, the efficacy of pelvic floor muscle training is limited in women with severe incontinence. Conservative methods have been shown to be time consuming and of variable efficacy (Berghmans et al., 1998).

Oestrogen replacement therapy with oestrogen or progestogens, which acts on the supporting tissue of the pelvic floor as well as increasing α-ARs density, vascularity/thickness, sensitivity, neuronal sensitivity and metabolism poses the question of safety (Csizmadi et al., 2004). Oestrogen replacement therapy is associated with breast cancer, ovarian cancer, and stroke. The FDA thereby mandated that all labels of oestrogen replacement medication must carry a warning stating the increased risks for heart disease, heart attacks, strokes and breast cancer. Moreover, continual use of oral oestrogen substitution has been stated to increase the risk of developing SUI (Goldstein et al., 2005; Sood et al., 2014). Thus, due to the lack of evidence of its effectiveness and increased awareness of the harms of hormone therapy (Sood et al., 2014), oral oestrogen is less prescribed as a treatment of SUI.

Pharmacological treatment of stress urinary incontinence

In women who are not candidates for conservative therapies or surgical interventions, pharmacological agents are often used off-label to alleviate the symptoms. Pharmacotherapy focuses on increasing maximal urethral closure pressure, that is, enhancing the resistance of the proximal urethra and the bladder neck. Alpha-AR agonists, such as pseudoephedrine and phenylephrine, are used off-label for SUI treatment based on the urethral smooth muscle response to stimulation and improvements in intrinsic sphincter deficiency.

Imipramine: The tricyclic antidepressant, imipramine, used off-label for the treatment of SUI, facilitates urine storage by several mechanisms. It decreases bladder contractility through an antispasmodic effect on the detrusor muscle, increasing outlet resistance through an α-adrenergic effect on the bladder neck (Cameron et al., 2013). It also antagonises mAChR, inhibits noradrenaline uptake into nerve terminals and smooth muscle. Moreover, imipramine mediated Ca^{2+}antagonism and histamine H_1 receptor blockade (Dave et al., 2002). Older animal studies and low-quality human trials have suggested a benefit in SUI and urgency urinary incontinence, but high-quality evidence is lacking. Currently, the use of imipramine for the management of SUI and urgency urinary incontinence lacks strong

clinical evidence and cannot be recommended. Dave et al., (2002) reported serious cardiovascular toxicity in the form of ventricular dysrhythmias and orthostatic hypotension for imipramine usage. Recently, Caldwell et al., (2016) reported from randomised and quasi-randomised trials that imipramine reduced the number of wet nights during treatment, but it does not have a sustained effect.

Fesoterodine (Antimuscarinic): Fesoterodine 8 mg improved severe urgency and symptom in urgency urinary incontinence patients (Dubeau et al., 2014; Ginsberg et al., 2013). In a 12-week, randomised, double-blind, placebo-controlled, multinational trial of men and women aged ≥18 years with overactive bladder symptoms including urgency urinary incontinence, fesoterodine 8 mg showed significantly superior efficacy vs. placebo as measured by reductions in urgency urinary incontinence episodes (Chapple et al., 2014b). However, in a randomised, double-blind, placebo-controlled, crossover study, using urethral pressure reflectometry, fesoterodine did not affect urethral pressure (Klarskov et al., 2014). Moreover, it did not significantly decrease the number of incontinence episodes in women with SUI. Thus, the study of Klarskov et al., (2014) does not support the use of antimuscarinics for SUI. Thus the effectiveness of fesoterodine in the management of SUI is still unclear.

Duloxetine: Duloxetine, a dual noradrenaline and serotonin reuptake inhibitor has been reported to improve SUI symptoms (Dmochowski et al., 2003). Duloxetine reduced incontinence episodes and increased micturition control (Redaelli et al., 2015). Data also support duloxetine's efficacy in women with severe SUI (Cardozo et al., 2004; Dmochowski et al., 2003; Millard et al., 2004). A study by Fink et al., (2008) on the effect of off-label duloxetine on men with SUI after prostate surgery, showed that 40 mg duloxetine twice daily was effective. When taking Duloxetine, the average use of incontinence pads decreased per day. Moreover, Cardozo et al., (2004) showed a significant improvement with duloxetine compared with placebo in incontinence episode frequency. At the conclusion of the 8-week study, 20% of duloxetine-treated women were no longer interested in surgery. Ayeleke et al., (2015) reported the combined therapy of duloxetine and pelvic floor muscle exercise. However, there were no statistically significant differences between women (with SUI, urgency urinary incontinence or mixed urinary incontinence) who received pelvic floor muscle therapy added to duloxetine and those who received duloxetine alone.

Discontinuations due to side effects were significantly more widespread with duloxetine (Cardozo et al., 2004; Dmochowski et al., 2003; Millard et al., 2004). Side effects leading to discontinuation of duloxetine included dry mouth, fatigue, nausea, constipation, and hyperhidrosis (Bump et al., 2008; Lin et al., 2008; Schagen Van Leeuwen et al., 2008; Vella et al., 2008). The FDA currently approves duloxetine for the treatment of depression. It failed FDA approval for the treatment of SUI due to poor efficacy and increased suicidal ideation and suicide in depressed patients (Cameron et al., 2013).

Alpha$_1$-AR agonists: The α_1-ARs are a promising drug target for increasing the urethral luminal pressure in stress urinary incontinent patients (Segev et al., 2015). However, only preliminary data on the use of the α_1-ARs agonist for treating the SUI is available.

a) ***Phenylpropanolamine (PPA)***

Collste & Lindskog, (1987) showed in a double-blind placebo-controlled study in 24 patients that phenylpropanolamine, 50 mg twice daily increased maximum urethral closure pressure. However, only 8-29% improvement in maximum urethral closure pressure was recorded. Complete continence was not achieved in this study. This effect was quite low compared to the expected maximum urethral closure pressure improvement targeted in patients. Ek et al., (1978) showed up to 55% maximum urethral closure pressure improvement using 100mg (norephedrine) instead, which increased the severity of side effects. Moreover, only 2 of 22 patient conditions improved. In a cross-over, blinded study in dogs, Segev et al., (2015) showed a significant increase in systolic, diastolic, and mean blood pressure following administration of phenylpropanolamine at 2 mg/kg body weight. Based on the cardiovascular side effect in human, phenylpropanolamine was withdrawn for use in humans in 2003 by the US FDA.

No increase in maximum urethral closure pressure with phenylpropanolamine was found in human (Obrink & Bunne, 1978) or beagle dogs (Noel et al., 2013); only one of 10 women reported improvement. The result reported by Obrink & Bunne, (1978) might be significantly affected by the loss of tissue response on long-term exposure (up to three weeks). White & Pomeroy, (1989) reported this response in female dogs with a decrease in a urethral response associated with recurrence of incontinence after a prolonged administration (three

months) of phenylpropanolamine. Furthermore, another pharmacokinetic and urodynamic study for continent female beagle dogs showed that urethral resistance increased after single daily phenylpropanolamine administration, but not when phenylpropanolamine was administered every 6 hours during the day (Noel et al., 2010). Desensitisation of the α-ARs has been proposed by several authors to explain this observation (Akinaga et al., 2013).

b) ***Other α_1AR agonists***

A61603, an α_{1A}-AR agonist, shows high specificity for α_{1A}- and α_{1L}-AR phenotypes and is essentially inactive at the α_{1B}- and α_{1D}-ARs (Yoshiki et al., 2013). A61603 was more potent than noradrenaline at the α_{1A}-AR phenotype and showed full agonist activity at both α_{1A} and α_{1L} phenotypes (Yoshiki et al., 2013). Systemic administration of A61603 in dog and conscious rat models elevated the intraurethral pressure (Knepper et al., 1995). To our knowledge, no report has used A61603 to increase the urethral luminal pressure in the human.

Methoxamine an α_1- AR agonist evoked non-significant increases in maximum urethral pressure but also caused a significant rise in blood pressure and significant fall in heart rate at maximum dosage in women (Radley et al., 2001). This study also reported systemic side effects including headaches, piloerection, and cold extremities in subjects.

The potential therapeutic benefits of the selective $\alpha_{1A/L}$-AR partial agonist Ro 115-1240 have also been investigated in women with mild-to-moderate SUI (Musselman et al., 2004). Clinical studies suggested that selective $\alpha_{1A/L}$-AR partial agonists have the potential to improve the symptoms of SUI with little or no cardiovascular effect (Blue et al., 2004; Musselman et al., 2004). In an in vivo model of urethral function, PF-3774076, a partial agonist at the human α_{1A}-AR resulted in a dose-dependent increase in urethral pressure in both the proximal and distal regions of the urethra (Conlon et al., 2009). However, further clinical applications of these drugs are lacking in the literature.

The present challenge in the use of α-AR agonists for SUI pharmacotherapy is the lack of exclusive selectivity for the urethral α-ARs, as severe cardiovascular adverse side effects such as headaches, elevated blood pressure, anxiety, tremor, weakness, respiratory difficulty are common (Ek et al., 1978; Segev et al., 2015). A better understanding of the pharmacology/physiology of urethral tissue is crucial to aid better drug development for SUI.

1.2 THE ANORECTUM

1.2.1 Structure

The rectum is a muscular channel (12-15cm) that stores stools and terminates with a 2-4cm long muscular tube called the anus (Figure 1.9). The internal anal sphincter (IAS), external anal sphincter, anal mucosa folds, as well as the vascular cushion surround the anal opening (Figure 1.9) (Stoker, 2009). The mucosa of the upper anal canal is lined by columnar epithelium while squamous epithelium lines the mucosa below the dentate line (Tanaka et al., 2012). Furthermore, the transition between columnar, transitional or squamous epithelium is present above the dentate line. The anal crypt is of importance in anorectal disorders because foreign materials may lodge there, thereby obstructing faecal passage and leading to sepsis. The mucosa folds and the vascular cushion mediate the tight sealing of the anus, thereby, helping to reinforce tone during voluntary squeeze (Gibbons et al., 1986a;b).

1.2.1.1 Internal Anal Sphincter (IAS)

The IAS is a 0.3-0.5cm thick extension of the circular smooth muscle of the rectum. It is tonically contracted and accounts for 80% to 85% of the anal canal resting pressure (Frenckner & Euler, 1975). Internal anal sphincter basal tone is inherently myogenic, although angiotensin II and prostaglandin $F_{2\alpha}$ have been shown to modulate the tone (De Godoy et al., 2004; De Godoy et al., 2009). This tone is dependent on extracellular Ca^{2+} entry via the L-type Ca^{2+} channels (Cook et al., 1999b). Moreover, IAS spontaneous basal tone contributes to the pathophysiology of numerous gastrointestinal disorders, such as anal fissures, constipation, haemorrhoids, and Hirschsprung's disease.

The IAS contracts in response to noradrenaline released by sympathetic innervation (Carlstedt et al., 1988). Internal anal sphincter relaxation is neurogenic and is mediated via non-adrenergic, non-cholinergic neurotransmitters such as NO and vasoactive intestinal peptide. Beta-ARs and peptidergic receptors also contribute but via mechanisms that are not entirely understood. Moreover, contraction and relaxation of the IAS are regulated by the actions of other cell types, including ICC and fibroblast-like cells (De Lorijn et al., 2005).

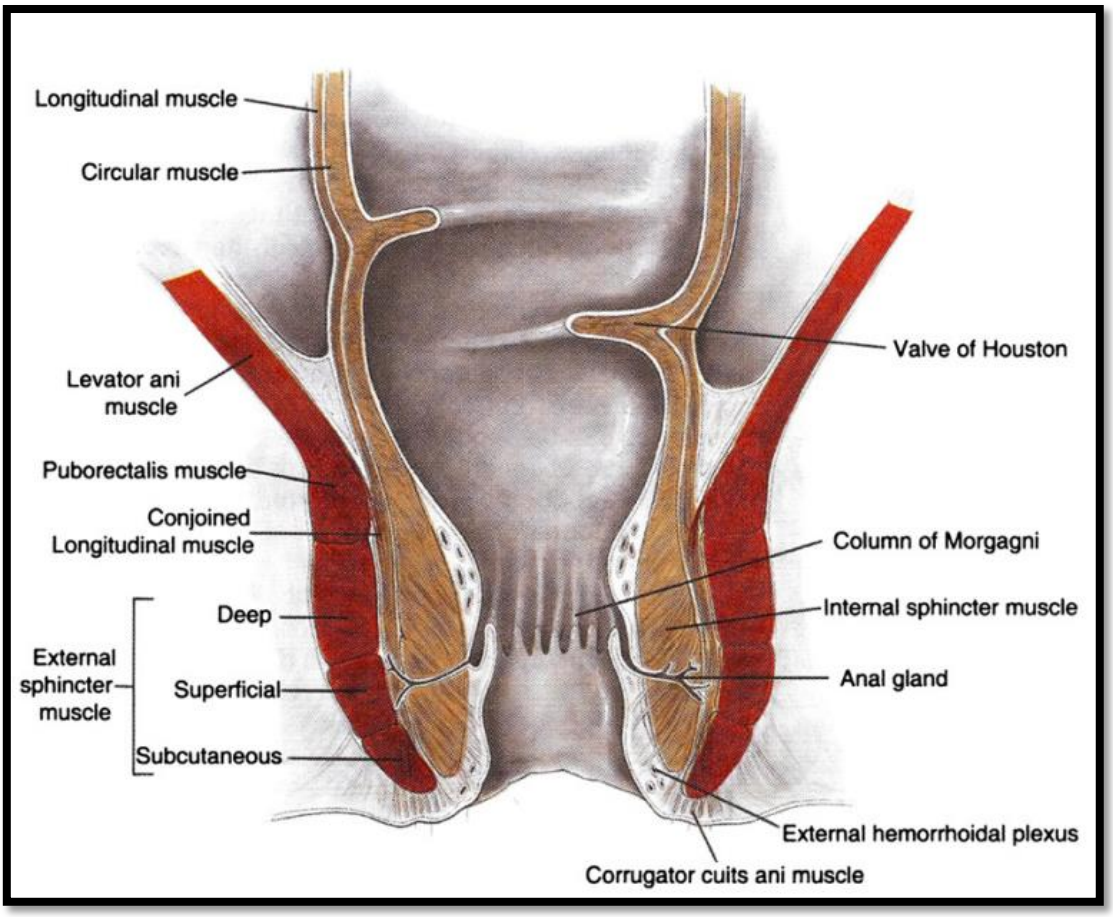


Figure 1.9 The anorectum. The anorectum is a channel that serves as the temporary store and passage for stools. The anorectum is made up of the internal anal sphincter, external anal sphincter; extension of the levator ani, puborectalis, and the anal cushion that helps to maintain faecal continence. Adapted from (Gordon & Nivatvongs, 2007).

1.2.1.2 External Anal Sphincter (EAS)

The external anal sphincter, a 0.6-1cm thick striated muscle contributes to the tonic contraction as well as the voluntary control of the anorectum. It is an extension of the levator ani muscle and morphologically separated from the IAS. A spinal reflex causes the external anal sphincter to contract during sudden intra-abdominal pressure increases, such as coughing, thereby, external anal sphincter helps to maintain continence (Kumar et al., 1990; Sun et al., 1990).

1.2.1.3 Puborectalis muscle

The puborectalis muscle is also a striated muscle which wraps around the rectum. It creates the anorectal angle (normally 60° to 105°) which slows the progress of stool to the anal canal. This muscle also prevents movement of faeces from the rectum to the anal canal between defecations. The puborectalis muscle functions with voluntary control and produces a tonic contraction (Fernandez-Fraga et al., 2002).

1.2.2 Innervation and function

The anorectum is innervated by the autonomic (sympathetic and parasympathetic nerves) and enteric nervous systems while the outer funnel-shaped tube of the anorectum has somatic innervation. The main innervation of the gastrointestinal tract is intrinsic, from neurones of the enteric nervous system. These innervations modulate peristalsis and are involved in the defecation reflex. The autonomic inputs to the IAS are tyrosine hydroxylase staining fibres (sympathetic) and NO staining fibres (parasympathetic) (Ishiyama et al., 2014; Kinugasa et al., 2014).

The proximal gastrointestinal tract (proximal-mid colon) receives extrinsic parasympathetic efferent innervation via the vagus nerve (Ranson & Saffrey, 2015). Extrinsic parasympathetic efferents to the distal bowel arise from spinal segments S_2–S_4 in humans and innervate the distal colon, anorectum, and IAS. The sympathetic innervation of the distal bowel originates in spinal segments T_5–L_2 and these thoracolumbar innervations inhibit bowel movement and maintain continence by contracting the IAS (Ranson & Saffrey, 2015). The sympathetic

hypogastric nerves of the IAS control involuntary activities of the IAS (Barleben & Mills, 2010). Moreover, the epithelium of the proximal region to the anorectal dentate line is innervated by the autonomic nervous system, while the somatic nervous system innervates the epithelium of the distal part of the anal canal (Wang et al., 2013). The epithelial fibres either have a sensory function, for example, those positive for calcitonin gene-related peptide and substance P or secretory function, for example, the vasoactive intestinal peptide (Wang et al., 2013).

The sacral spinal cord accommodates the motoneurons that regulate the voluntary response of the pelvic floor muscles and external anal sphincter during defecation (Ranson &Saffrey, 2015). The external anal sphincter has somatic innervation from the pudendal nerve (S_2–S_4). Moreover, the puborectalis muscle has somatic innervation from the pelvic branches of the S_3 and S_4 pudendal nerves. The pudendal nerve modulates sensation in the perianal and genital skin, the tone of the external anal sphincter and rectoanal contractile reflexes (Frenckner & Euler, 1975).

1.2.2.1 Sympathetic innervation of the internal anal sphincter

IAS contraction occurs through sympathetic nerve stimulation (Moszkowicz et al., 2012). Autoradiographic studies with [^{3}H] prazosin showed a positive association of α_1-AR with immunohistochemically identified smooth muscle fibres of sheep and human IAS (Rayment et al., 2010). The sheep IAS also showed a positive result to adrenergic nerves staining using a tyrosine hydroxylase antibody (Rayment et al., 2010).

Oral administration or intravenous infusion of α-AR antagonist caused a reduction in anal sphincter pressure (Gutierrez & Shah, 1975; Pitt et al., 2001) suggesting a role for α-ARs in the tonic control of sphincter pressure. Likewise, adrenergic stimulation via intravenous infusion of α-and β-AR agonists in cats (Penninckx et al., 1973) and humans (Gutierrez & Shah, 1975) has demonstrated that the effects of α-AR stimulation are excitatory to the IAS and that β-AR effects are inhibitory. The selective α_1-AR antagonist prazosin reduced hypogastric nerve induced elevation of resting pressure of the sphincter in anaesthetised opossum (Yamato & Rattan, 1990). Moreover, exogenous addition of phenylephrine increases IAS resting pressure in opossum, an effect blocked by prazosin. Tetrodotoxin did

not modify this effect and was consistent with a post-junctional location for α_1-ARs on smooth muscle cells (Yamato & Rattan, 1990). The importance of α-ARs in IAS tone is underlined by the use of L-erythro-methoxamine (α-AR agonist) to elevate mean anal resting pressure of patients with faecal incontinence (Nisar et al., 2005). Moreover, radioligand binding studies and immunohistochemistry have revealed the presence of α_{1A}-ARs and α_{2D}-ARs binding sites in the sheep IAS (Rayment et al., 2010). Activation of the α_1-AR subtype mediates contraction of IAS smooth muscle of pig (Mills et al., 2008), sheep (Rayment et al., 2010) and human (Owaki et al., 2015). The α_{1A}-AR subtype predominantly contributes to human IAS contraction (Owaki et al., 2015) and specifically the α_{1L}-AR subtype in the porcine IAS (Mills et al., 2008).

Alpha$_2$-ARs are known to be present in the duodenum and proximal colon (Zhang et al., 1992; Valet et al., 1993) and α_2-AR agonists induce colonic and rectal relaxation (Viramontes et al., 2001; Camilleri et al., 2003). Radioligand binding studies have revealed the presence of α_2-AR binding sites in the sheep anal sphincter membranes, but the density was only half of that detected for α_1-AR binding sites (Rayment et al., 2010). Activation of α_2-AR inhibits non-adrenergic, non-cholinergic -mediated recto-anal inhibitory reflex-induced IAS relaxation of opossum IAS (Yamato & Rattan, 1990). The α_2-AR agonists induced contraction in the sheep IAS (Rayment et al., 2014). Rayment et al., (2014) also provided evidence for a dual role for α_2-ARs on the sheep isolated IAS: direct constrictor effect and modulation of noradrenergic transmission. Also, α_2-ARs played a role in noradrenaline-mediated constriction of the pig isolated IAS, but only at high concentrations (Mills et al., 2008).

The inhibitory effect of sympathetic nerve stimulation in the human gastrointestinal tract is mediated by the activation of post-junctional β-ARs (Manara et al., 2000). The classical β_1-ARs, β_2-ARs, and β_3-ARs mediate these responses. Beta$_2$-ARs activate both $G_{s\alpha}$ and $G_{i/o}$ α-protein subunits and induce relaxation in the rat IAS via both cyclic AMP/cyclic GMP pathways. In contrast, β_1-ARs / β_3-ARs activation caused smooth muscle relaxation via the $G_{i/o}\alpha$- guanylate cyclase/cyclic GMP/protein kinase G pathway (Li et al., 2004). The β_3-AR-mediated effect was mediated via the cyclic GMP pathway similar to NO (Banwait & Rattan, 2003). Agonists acting at all three β-ARs relaxed the opossum IAS (Rathi et al., 2003). Ballester et al., (2010) reported a β_3-mediated relaxation of sphincter tone of human IAS.

Direct β_3-relaxation appears smaller than that obtained for nonselective agonists, however, β_3-AR activation does mediate IAS relaxation.

1.2.2.2 Parasympathetic innervation of the internal anal sphincter

Cholinergic neurones mediate the contraction of gastrointestinal smooth muscle (Inoue & Isenberg, 1990). Acetylcholine binding to mAChRs (M_2, M_3) on canine colonic circular smooth muscle mediates increases in cytosolic Ca^{2+} and contraction (Zhang et al., 1991). Likewise, bethanechol induced contraction of the human IAS acting through M_3 mAChRs (Singh et al., 2009). However, in some studies, acetylcholine and carbachol have been shown to exert inhibitory effects on IAS smooth muscle (Burleigh et al., 1979; Ramalingam et al., 2010). According to Ramalingam et al., (2010), the carbachol caused relaxation of IAS strips via two mechanisms, which includes the release of noradrenaline which might activate β-AR because guanethidine (10μM) blocked the carbachol responses. Secondly, the report suggests that mAChR were present on NO-releasing nerves such that the activation of mAChR mediated the release of NO, because inhibitors of NOS attenuated the response to carbachol (O'Kelly et al., 1993b; Ramalingam et al., 2010).

1.2.2.3 Sensory innervation of the internal anal sphincter

The sensory nervous system analyses the exact contents of the rectum and produces the sense of urge. Anal sensations are necessary for a person to contract the external anal sphincter if defecation is to be postponed. Both unmyelinated and myelinated afferent nerve fibres are present in the anorectum (Bharucha, 2006) and mediate the stretch or distension-induced sensory responses. They also induced the viscero-visceral, the recto-anal inhibitory and the recto-anal contractile reflexes (Remes-Troche et al., 2010). Sensory fibres transmit the sensation of rectal distention in the S_2-S_4 pudendal nerves (Rogers, 1992). Pudendal nerve block creates a loss of rectoanal contractile reflexes and sensation in the perianal and genital skin.

The function of sensory nerves of the anal canal is to allow anal sampling. The anal sampling reflex, including short-lasting rectal contraction and relaxation of the upper part of the anal canal, allows the subject to sense the content of the rectum. Rectal sensation arises from the

stimulation of nerve endings and mechanoreceptors, both in the rectal wall and the adjacent pelvic structures (Rogers, 1992). This is supported by the finding of existence of intraganglionic laminar nerve endings in the myenteric plexus of the rat rectal wall that are sensitive to mechanical distension (Zagorodnyuk et al., 2005).

1.2.2.4 Enteric Nervous System (ENS)

The enteric nervous system 'the second brain' works in concert with neural control centres in the lower brain stem, spinal cord and sympathetic ganglia (Furness, 2015). The enteric nervous system consists of the network of ganglia (myenteric plexus) and the smaller ganglia (the submucous plexus) (Furness, 2015). The myenteric plexus lies between the rectal wall smooth muscle layers, the longitudinal and circular muscle layers which make up the muscularis externa. However, the smaller ganglia, the submucosal plexus, are present in the connective tissue that lies between the muscularis externa and the mucosa.

The mechanosensitive neurones have been described in the myenteric plexus using extracellular electrodes and within the gut, the enteric nervous system can sense mechanical stimuli and trigger gut reflex behaviour (Mazzuoli & Schemann, 2012). Mazzuoli & Schemann, (2009) presented evidence for multifunctionality of rapidly adapting mechanosensitive enteric neurones which may fulfil sensory, integrative and motor functions.

The roles of the enteric nervous system include control or regulation of movements of the digestive tract and release of gastrointestinal hormones. The enteric nervous system controls the complex functions of the gastrointestinal tract such as peristalsis, blood flow, independent regulation of motility and secretion (Benarroch, 2007; Brehmer, 2006; Lake & Heuckeroth, 2013; Wood, 2010; Zhang & Hu, 2013). Moreover, the distension induced motor behaviour of the isolated intestine is regulated by the enteric nervous system (Mazzuoli & Schemann, 2012).

1.2.2.5 Stool Storage/Expulsion

When an individual is not defecating the rectum is compliant. During faecal storage, the rectum receives increasing amounts of stool volume while maintaining a constant intraluminal pressure. The rectum can accommodate up to 300 ml of faecal material before

intraluminal pressure increases and a feeling of urgency develops. The IAS relaxes temporarily in response to rectal distension and this relaxation reflex, mediated by enteric nerves, is not under voluntary control. When a stool accumulates in the rectum, the rectum distends (compliance), and the IAS relaxes while the external anal sphincter contracts. The external anal sphincter contraction helps to perceive and differentiate between a hard stool, soft stool and gas (anal sampling) (Miller et al., 1988a), which is frequently abnormal in incontinent patients (Miller et al., 1988b). If the environment is not conducive for defecation, faecal materials can be returned to the colon for more water reabsorption. If a patient chooses not to defecate, they increase contraction of the external anal sphincter to maintain continence. Some stool also moves slightly more to the proximal colon, thereby reducing the pressure in the rectum as well as the urge to defecate.

When the environment is conducive for passing out the stool, defecation is initiated. Initiation of defecation is associated with activation of the stretch receptors in the rectum, as well as initiation of the recto-anal inhibitory reflex (Scharli & Kiesewetter, 1970). Neurally-mediated relaxation of the IAS muscle is mediated by distension sensed by mechanoreceptors of the rectal wall. To defecate, a suitable posture is assumed, the rectal angle straightens, the sphincters relax, the pelvic floor descends, the rectum contracts and abdominal pressure rises due to straining. The pelvic floor then descends and the rectum contracts, the external anal sphincter relaxes while the tonic activity of the external anal sphincter is inhibited. Together, these events lead to the evacuation of the rectal contents (Bartolo et al., 1985). Any activity or damage that affects the processes described could cause or increase the risk of faecal incontinence.

1.2.3 Faecal incontinence (FI) and anal fissures

Faecal incontinence is defined as a complaint of involuntary loss of flatus and/or liquid or solid stool via the anus. The prevalence of faecal incontinence varies between 5% and 15% of the general population (Damon et al., 2006; Macmillan et al., 2004). The prevalence increases with age from approximately 3% in people in the second decade to 16% in people aged 70 years or over (Ditah et al., 2014). US community-based prevalence data suggest that faecal incontinence affects approximately 20 million adults (Whitehead et al., 2009). A cross-sectional survey conducted in a primary care setting in Sydney, Australia, reported a

prevalence of 12.1% for faecal incontinence (Ng et al., 2015). However, these figures are most probably underestimated, since less than 25% of patients with faecal incontinence report it to their physician.

The incidence of faecal incontinence increases in the frail, immobile, elderly and patients with severe behavioural, neurological and obstetric disorder/injury (Ditah et al., 2014; Shah et al., 2012). Chronic inflammatory bowel diseases and side effects of radiotherapy can also lead to faecal incontinence (Table 1.3). In addition, high body mass index, smoking, diarrhoea and constipation are all associated with faecal incontinence (Ditah et al., 2014; Townsend et al., 2013). Faecal incontinence is a cause of significant cost due to diagnostics, treatment, care and reduced ability to work (Xu et al., 2012). The average annual total cost for faecal incontinence management was $4110 per person annually in the United States in 2010 (Xu et al., 2012).

Faecal incontinence can be classified as urge, passive, overflow or post-defecatory. Urge faecal incontinence is the need to pass faecal material, but the stool being released before getting to the convenience, due to lack of voluntary control. It is associated with dysfunction of the external anal sphincter (Engel et al., 1995). On the other hand, passive faecal incontinence is a situation where the individual is aware of faecal leakage only after the faecal material has been released. Moreover, overflow faecal incontinence is related to leakage of stools when the rectum is blocked by a hard stool, incomplete evacuation, and impaired rectal sensation is responsible (Engel et al., 1995; Rao et al., 2004). Post defecatory faecal incontinence is characterised by faecal leakage following passing out faecal material. Post defecatory faecal incontinence is usually associated with an inability to clear the rectum as well as a loss of proper function of the IAS (Engel et al., 1995; Vaizey et al., 1997).

Faecal incontinence is prevalent due to thinning of the external anal sphincter, degeneration of the IAS, weakening of the muscular contraction and increased latency of the pudendal nerve firing (Lubowski et al., 1988; Santoro et al., 2012; Tetzschner et al., 1995). Useful examinations for faecal incontinence include anal endosonography to detect any damage to the anal sphincters and anorectal manometry to measure compliance and rectal sensation as well as pelvic floor muscle strength (Mahony et al., 2004).

Table 1.3 Common Causes of faecal incontinence and predisposing factors

Common Causes of faecal incontinence	Predisposing factors	References
Anal sphincter dysfunction	Congenital, anorectal malformations, radiation therapy, obstetric anal sphincter injury, anal surgery, perianal fistulas, sexual abuse.	Alsadius et al., 2012.
Neurological disorders	Spinal cord lesions, stroke, multiple sclerosis, diabetic neuropathy, obstetric nerve damage.	Bytzer et al., 2001; Chia et al., 1995; Lunniss et al., 2004.
Rectal disorders	Inflammatory bowel disease, radiation therapy, rectal prolapse, faecal impaction.	Bharucha et al., 2015.
Psychological	Dementia.	Bharucha et al., 2015.
Myopathy	Systemic scleroderma.	Fynne et al., 2011.

1.2.3.1 Treatments for faecal incontinence

Conservative therapy is the first-line treatment for patients with faecal incontinence. Personal hygiene and diet control, the use of antidiarrheal drugs (loperamide, codeine) and pelvic floor therapy characterise conservative treatment (Palmer et al., 1980). Constipating agents decrease intestinal motility and stool frequency. Stool-bulking agents, such as mucilage, are also useful in the management of faecal incontinence with loose stool because they improve stool consistency by increasing water absorption by stools (Table 1.4). Even though the efficacy of synthetic fibres has not been rigorously proved scientifically, mucilages are still recommended in clinical practice (Vitton et al., 2014), particularly combined with other conservative treatments (Sjodahl et al., 2015). However, this therapy is useful only in the patient with low volume loose-stool associated faecal incontinence (Meyer & Richter, 2016). In regards to antidiarrheal drugs, Markland et al., (2015) reported that the usage of loperamide induces chronic constipation in patients in a randomised double-blind placebo-controlled crossover trial. Thus, antidiarrheal drugs are not the best option for treating faecal incontinence.

Surgery, pelvic floor muscle exercise, and electrical stimulation are the next level of treatment (Leung et al., 2006; Pager et al., 2002). The data on pre- and post-treatment comparisons of pelvic floor muscle exercises for faecal incontinence are less well established compared with urinary incontinence. Anorectal surgery is associated with complications such as bleeding and infection (Kunitake & Poylin, 2016). The most feared long-term complications of surgery include faecal incontinence, anal stenosis, and chronic pelvic pain (Kunitake & Poylin, 2016).

A double-blind randomised trial applying topical oestrogens in postmenopausal women with faecal incontinence showed improvement of continence (Pinedo et al., 2009). Pinedo et al., (2012) also reported in a randomised double-blind trial that novel zinc-aluminium ointment decreases faecal incontinence significantly compared with placebo. Aluminium ions induce cytoplasmic Ca^{2+} oscillation, most probably by acting on the plasma membrane and mediating Ca^{2+}influx into smooth muscle cells via the L-type Ca^{2+}channels (Himpens et al., 1991).

Table 1.4 Faecal incontinence and anal fissure; present treatments, adverse effects, and limitations

Anorectum disorders	Present prescriptions and treatments	Side effects/ limitations	References
Anal fissure.	Topical nitrates; they are nitric oxide precursor (nitroglycerin ointment). They mediate internal anal sphincter relaxation.	Headaches and lightheadedness.	Brisinda et al., 1999; Jonas et al., 2001; Pitt et al., 2000; 2001; Ram et al., 2007.
	Calcium channel blockers such as nifedipine, diltiazem which mediate internal anal sphincter relaxation.	Mild incontinence to flatus and stool. Headache, nausea or vomiting.	
	Botulinum toxin (BT) injections; blocks neuronal release.	Increased urinary residual volume, heart block, skin and allergic reactions, muscle weakness, postural hypotension as well as recurrence of anal fissure & cost.	
	Alpha$_1$ antagonist; inhibits α_1-ARs.	Poor healing rate and fissure recur (indoramin).	
	Surgery such as anal stretch/dilation and lateral sphincterotomy.	Damage to the IAS with increased risk of incontinence.	
Faecal incontinence.	The α-AR agonists such as phenylephrine.	Stinging sensation.	Cheetham et al., 2001; Malouf et al., 2001; Palmer et al., 1980; Siproudhis et al., 2007; Sung et al., 2007.
	Constipating agents such as loperamide, atropine sulphate, diphenoxylate, difenoxin, and codeine. Low fibre diet; are prescribed in mild cases.	Constipation and abdominal pain (loperamide), abdominal pain (codeine), and nausea (atropine sulphate).	
	Incontinence pad, anal plug/ electrical stimulation are prescribed in mild cases. Surgical repair; are prescribed for patients with the damaged external anal sphincter.	Cost.	
	Physical therapy, pelvic floor muscle exercise.	Time-consuming.	
	Bulking agents.	Post-operative complications (e.g. infection, bleeding, injection site or anal pain or discomfort, evacuation difficulty).	

Aluminium ions increase the amplitude of K^+-induced contraction and mediate changes in the physical properties of the plasma membrane and the sarcoplasmic reticulum. They can also influence actomyosin ATPase and produce structural changes to the muscle-actomyosin complex (Davidovskaya & Bogutska, 2003; Himpens et al., 1991). Although Pinedo et al., (2012) showed that zinc-aluminium ointment decreases faecal incontinence, the issue of risk of cancer and other complications needs consideration.

Topical administration of an α_{1A}-AR agonist, L-erythro-methoxamine 3% (w/w) gel (higher α_{1A}-AR activity than phenylephrine) applied to the anal canal or rectum of healthy volunteers increased maximum anal resting pressure for 5 hours. L-erythro-methoxamine 3% (w/w) gel also elevated systemic blood pressure in some healthy volunteers (Nisar et al., 2005). Moreover, when lower concentrations of L-erythro-methoxamine (0.3% or 1% (w/w) were used the same result (3%) was reported (Nisar et al., 2007). Recently, Simpson et al., (2014) reported in a randomised controlled trial, an increase in anal sphincter tone with the use of NRL001 (one of four possible stereoisomers of methoxamine). There was a dose-dependent increase in anal sphincter tone when comparing the 5 and 10 mg doses of NRL001. However, the 15 mg dose did not have a significantly greater effect than the 10 mg dose. The inability of methoxamine to maintain the resting pressure for longer hours may suggest desensitisation of α_1-ARs to this agonist.

Siproudhis et al., (2014) used NRL001 in phase II double-blind randomised trial and evaluated the efficacy, safety, and tolerability of locally applied NRL001 in the patients with faecal incontinence. However, the results are yet to be published. Bell et al., (2014a) further reported in another double-blind, placebo-controlled, randomised trial that NRL001 was generally well tolerated over 14 days with a daily dosing. Likewise, plasma NRL001 did not accumulate over recorded time. Treatments caused changes in vital sign, most notably decreased heart rate and other adverse events commonly reported with NRL001 treatment were events indicative of a systemic α-adrenergic effect. Moreover, Bell et al., (2014b) did report in another randomised, controlled, crossover study that administration of NRL001 suppositories led to decreases in heart rate when compared with placebo data. NRL001 was found to have a dose-dependent effect on heart rate; however clinically relevant bradycardia was not reported (Gruss et al., 2014).

Thus, topical agents such as phenylephrine (Badvie & Andreyev, 2005; Carapeti et al., 2000a; Park et al., 2015) have shown some efficacy for faecal incontinence. However, given the limited number of studies and the potential adverse effects such as headaches and dermatitis, these treatments are not recommended in clinical practice.

1.2.3.2 Anal Fissures

A typical anal fissure is a tear/ulcer in the anoderm distal to the dentate line, which usually causes pain during and after defecation (Hananel & Gordon, 1997 ; Madalinski, 2011). In contrast, an atypical anal fissure is characterised by multiple ulcers located in locations other than the anoderm and could be associated with Crohn's disease, ulcerative colitis, anal cancer, tuberculosis, HIV, syphilis, herpes, and leukaemia (Herzig & Lu, 2010; Nelson et al., 2012). When a tear in the anoderm heals within six weeks, it is considered as an acute anal fissure, while if extended beyond six weeks, it is regarded as a chronic anal fissure (Madalinski, 2011). Features of a chronic fissure are exposed fibres of IAS at the base, hypertrophied anal papilla proximally, and a skin tag or sentinel pile distally (Brown et al., 1989). The exact cause of an anal fissure is not entirely clear, but it is thought to result from trauma to the anal canal. The trauma includes those to the anoderm during the passage of hard or large bowel movements, local irritation from diarrhoea, anorectal surgery, and anoreceptive intercourse (Beaty & Shashidharan, 2016; Markland et al., 2016). Males and females have an equal frequency of anal fissures. Fissures are commonly seen in middle-aged and younger patients, with a mean age of onset 39.9 years (Hananel & Gordon, 1997).

Increased pressure within the anal canal is a common characteristic of fissures. Studies have shown that the resting pressure of the IAS is higher in patients with fissures compared with normal controls (Garg, 2010; Nzimbala & Bruyninx, 2007). Hypertonicity of the anal sphincter is also responsible for some of the pain and spasm experienced with defecation, and it also has a deleterious effect on wound healing by reducing blood flow to the traumatised anoderm (Klosterhalfen et al., 1989).

Symptoms associated with acute fissures include anal pain, spasm, and/or bleeding with defecation (Hananel & Gordon, 1997). The pain is sharp or tearing and associated with defecation. The pain may also be present only during the defecation or last for several

minutes to hours after defecation. Bleeding is also a common symptom of anal fissure (Hananel & Gordon, 1997).

Present treatments for chronic anal fissure include surgery and conservative methods (consumption of fibre and warm water bath). The majority of acute anal fissures resolve without surgical intervention. Non-operative management of anal fissures includes alleviation of constipation and straining and relaxation of the IAS to improve blood flow and allow healing. Prescription of sitz baths, psyllium fibre supplementation and the use of stool softeners is a non-operative management.

However, when chronic fissures develop, healing is harder to achieve, thereby surgery is prescribed. Surgical methods, used to cause a therapeutic tear in the anorectum to correct chronic anal fissure, include anal stretch, open or close lateral sphincterotomy and dermal flap coverage of the fissure (Nelson et al., 2012). Although the gold standard for treating chronic anal fissure is surgical methods, they predispose the patient to the development of faecal incontinence (Poh et al., 2010). Hence, pharmacotherapy will be more appealing to many patients.

Present pharmacological approaches to chronic anal fissure include the use of topical nitrates (glyceryl trinitrate) (Scholefield et al., 2003), which release NO to relax the IAS (Berndt et al., 2004). Furthermore, Ca^{2+} channel blockers such as nifedipine (Cook et al., 1999a), blockers of acetylcholine release and botulinum toxin, which block nerve activity in the IAS, have been used for treating chronic anal fissure (Antropoli et al., 1999; Chrysos et al., 1996; Jost, 1997). Recently, Gandomkar et al. (2015) reported a recent randomised trial of 99 patients where lateral internal sphincterotomy was combined with botulinum toxin A injection and topical diltiazem in the treatment of chronic fissures. Overall healing rates were 65% in the botulinum toxin A-diltiazem group and 94% in the lateral internal sphincterotomy group. However, there was no statistical difference between these groups.

Nelson and colleagues reported the use of agents for anal fissure treatments in randomised controlled trials of nonsurgical therapies (Nelson, 2006; Nelson et al., 2012). These agents include nitroglycerin ointment (NO donor), isosorbide dinitrate, botulinum toxin, diltiazem, nifedipine, hydrocortisone, lignocaine, bran, minoxidil, indoramin, and placebo.

Nitroglycerin was found to be marginally but significantly better than placebo in healing anal fissure (Nelson, 2006; Nelson et al., 2012). However, late recurrence of the fissure was common. The dose of nitroglycerin has been studied extensively by Bailey et al., (2002) & Carapeti et al., (1999b). Carapeti et al., (1999b) reported healing in 67% patients treated with nitroglycerin compared with 32% with placebo in a randomised controlled trial with 0.2% nitroglycerin three times daily, or nitroglycerin starting at 0.2% with weekly 0.1% increments to a maximum of 0.6%. Headaches were reported by 72% of patients on nitroglycerin compared with 27% on placebo. However, of the fissures healed 25% to 33% of those healed with nitroglycerin recurred. Significant recurrence of symptomatic fissures and a high incidence of headaches are limitations of the treatment. Moreover, a report from Bailey et al., (2002) randomised, double-blind study and Scholefield et al., (2003) double-blind randomised controlled trial of intra-anally applied nitroglycerin ointment showed no difference in fissure healing among treated and placebo group. Thereby, they concluded that nitroglycerin ointment did not alter healing but significantly and rapidly reduced the pain associated with chronic anal fissures.

Although present pharmacological drugs seem to alleviate the pain of anal fissure, they are less effective than surgery, with diminished healing rates and severe adverse effects such as headaches and light-headedness, which discourages users and suggests a need for targeted specific drugs for the treatment of this condition.

Aim and objectives

Faecal incontinence and stress urinary incontinence are common conditions severely affecting the quality of life (QOL) of over 3 million Australians and which need better treatment options. The number of patients suffering from these conditions will increase as the ageing population increases and as a result of an increase in life expectancy due to the availability of better treatment for chronic diseases.

Presently, there are no effective treatments for incontinence and anal fissure. Current pharmacotherapy is non-specific to the urethra and the anorectum, and has limited success, a number of systemic side effects, low efficacy and morbidity (Bell et al., 2014a; Carapeti et al., 1999b; Nelson, 2006; Nelson et al., 2012). Although there is some understanding of the factors that help to maintain continence in the urethra and the anorectum, a greater understanding of the receptors, neurotransmitters involved and the intracellular signalling within the urethra and anorectum smooth muscle is needed. To develop more effective treatments, a better understanding of the physiology and pathophysiology of the urethra and anorectum is required; in particular the smooth muscle component, which is densely populated by α_{1A}-ARs and is responsible for maintaining continence in the bladder neck/urethra and also in the anorectum. Ultimately, the findings of the study may help identify novel drug targets and in the long-term the development of more effective pharmacotherapies for these critical conditions.

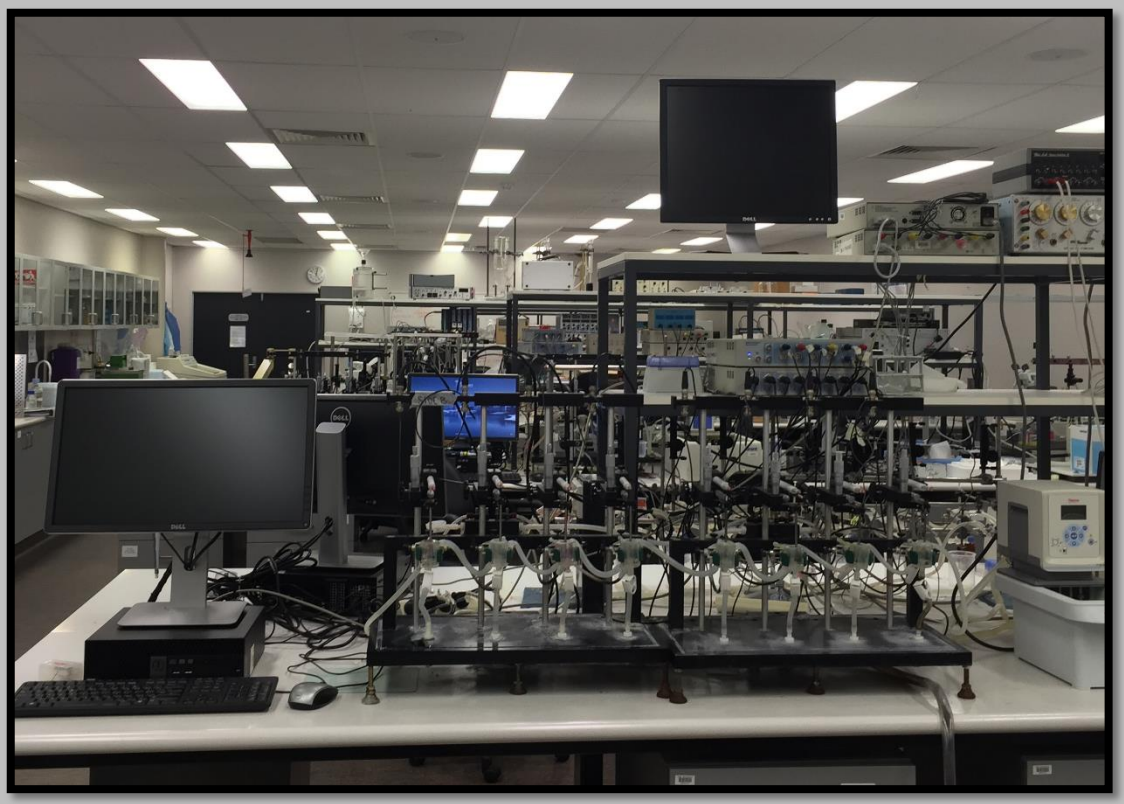
Aim: To elucidate the pharmacology of the urethral circular smooth muscle and IAS using the large White-Landrace pigs.

Objectives:

- To investigate the effect of age and the influence of the urothelium on receptor-mediated responses in the urethra.
- To compare the receptor-mediated responses in different regions of the urethra.
- To investigate potential desensitisation of receptor-mediated responses in urethral tissues.

- To investigate the postsynaptic interaction between the adrenergic and muscarinic system in the urethra.
- To investigate the neurotransmitters involved in modulating internal anal sphincter basal tone and contractility.

CHAPTER 2



2 MATERIALS AND METHODS

2.1 TISSUE SOURCE

For this project, porcine tissues were used as a model for human. Porcine tissues from the lower urinary tract and anorectum have been shown to be a good model for human, since pigs have similar physiology, autonomic pharmacology and functional properties to humans (Crowe & Burnstock, 1989; Brading et al., 2001; Burdzinska et al., 2012; Lentle et al., 2015; Mills et al., 2008; Sellers et al., 2000). In particular, the female pig urethra possesses many characteristics similar to that of the human female urethra (Greenland et al., 1996). Pig is often used as a suitable anorectal model of humans, being of a similar size. The porcine IAS is both qualitatively and quantitatively similar to human IAS tissue.

2.2 ETHICS

This project did not require ethical approval. All porcine tissues were collected from a local abattoir as remnants from animals being slaughtered for human consumption.

2.3 ANIMALS

Tissues from young (average age 6 months) female non-parous bacon pigs and aged parous female sows (average age 36 months) were used. The mean body weight of young non-parous bacon pigs was 80kg, while the mean body weight of aged parous sows was ≥180kg. The samples were from large White-Landrace pigs.

2.4 FUNCTIONAL TISSUE BATH TECHNIQUE

Fresh pig tissues collected from a local abattoir were immersed in fresh cold Krebs-bicarbonate buffer while being transported to the laboratory. In the laboratory, these fresh tissues were placed on a dissecting board in ice-cold Krebs bicarbonate (NaCl 118.4 mM, $NaHCO_3$ 24.9 mM, $CaCl_2$ 1.9 mM, $MgSO_4$ 2.41 mM, KCl 4.6 mM, KH_2PO_4 1.18 mM and d-glucose 11.7 mM), and with a sharp razor and dissecting scissors the urethra and the anorectum were dissected, so as to isolate the urethral circular smooth muscle (Figure 2.1A-C)

and the IAS of the anorectum (Figure 2.2A-D). The urethra was divided into proximal (2-3cm from the bladder neck), mid (3-5cm from the bladder neck) and distal, which is the remaining part up to the external orifice (Figure 2.1). The urethral regions were cut open, and strips of (2cm x 0.5cm) the circular smooth muscle were dissected out. When the urothelium/lamina propria was removed, this was achieved using fine dissection scissors.

For the anorectum, the tissue was cut open as shown in Figure 2.2A-B with scissors. The epithelium was carefully removed, and circular smooth muscle IAS strips (1.5cm x 0.3cm) were removed (Figure 2.2C-D) with a fine dissecting scissors. Tissues were set up in EZ-Bath tissue baths (Global-Town Microtechnology, Sarasota, FL, USA) containing Krebs-bicarbonate solution at 37°C and gassed with 5% CO_2 in oxygen and attached with a surgical thread to force transducers (MCT050/D, ADInstruments, Castle Hill, Australia) (Figure 2.3). The tensions developed by tissues were recorded using a PowerLab system using LabChart software (version 7) (ADInstruments).

Both urethral and IAS tissues were mounted under 100-250 milliNewton (mN) resting tension and allowed to equilibrate for 30-60 minutes, during which they were washed with the fresh Krebs-bicarbonate solution every 15 minutes, before the commencement of the experimental protocol.

2.5 PHARMACOLOGICAL PROTOCOLS

For pharmacological studies, adjacent pieces of tissues were dissected, set up in triplicate under identical conditions, and allowed to equilibrate. After equilibration, one tissue was incubated with the inhibitor for 30 minutes, the other served as a control in the absence of any inhibitor, while the third served as a vehicle control. Tissues were contracted to cumulative concentrations of agonist and responses compared with control tissues.

To investigate the effect of the urothelium/ lamina propria on receptor-mediated responses, the urothelium/lamina propria was carefully dissected off and separated from the smooth muscle. The tissues, with and without urothelium/lamina propria, were set up in pairs and cumulative concentration-response curves to agonists were constructed. Where the effects of inhibitors were investigated, these were incubated for 30 minutes after equilibration.

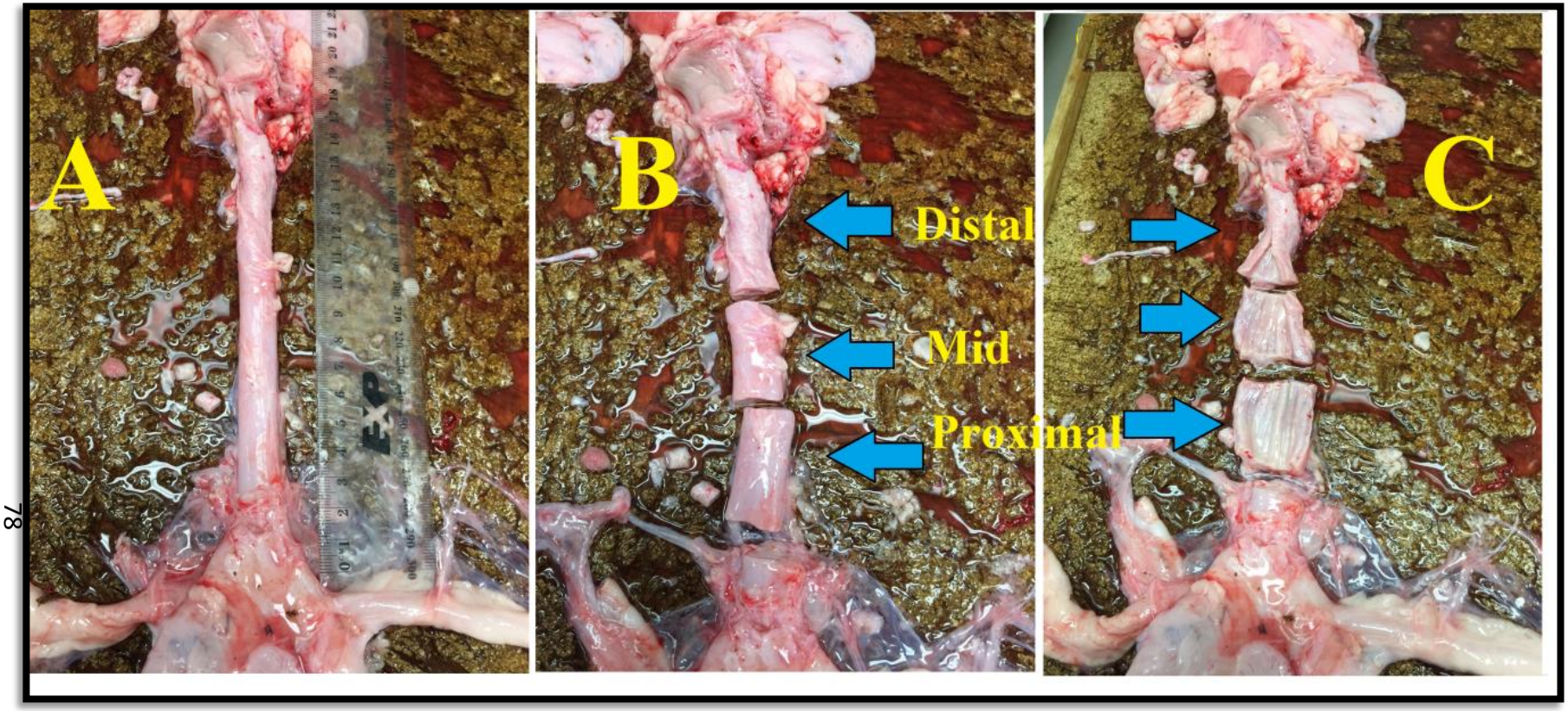


Figure 2.1 Dissection of the porcine urethra into regions. The urethra was dissected into three regions; the proximal, mid and distal urethra (B), and strips dissected from the opened tissues (C).

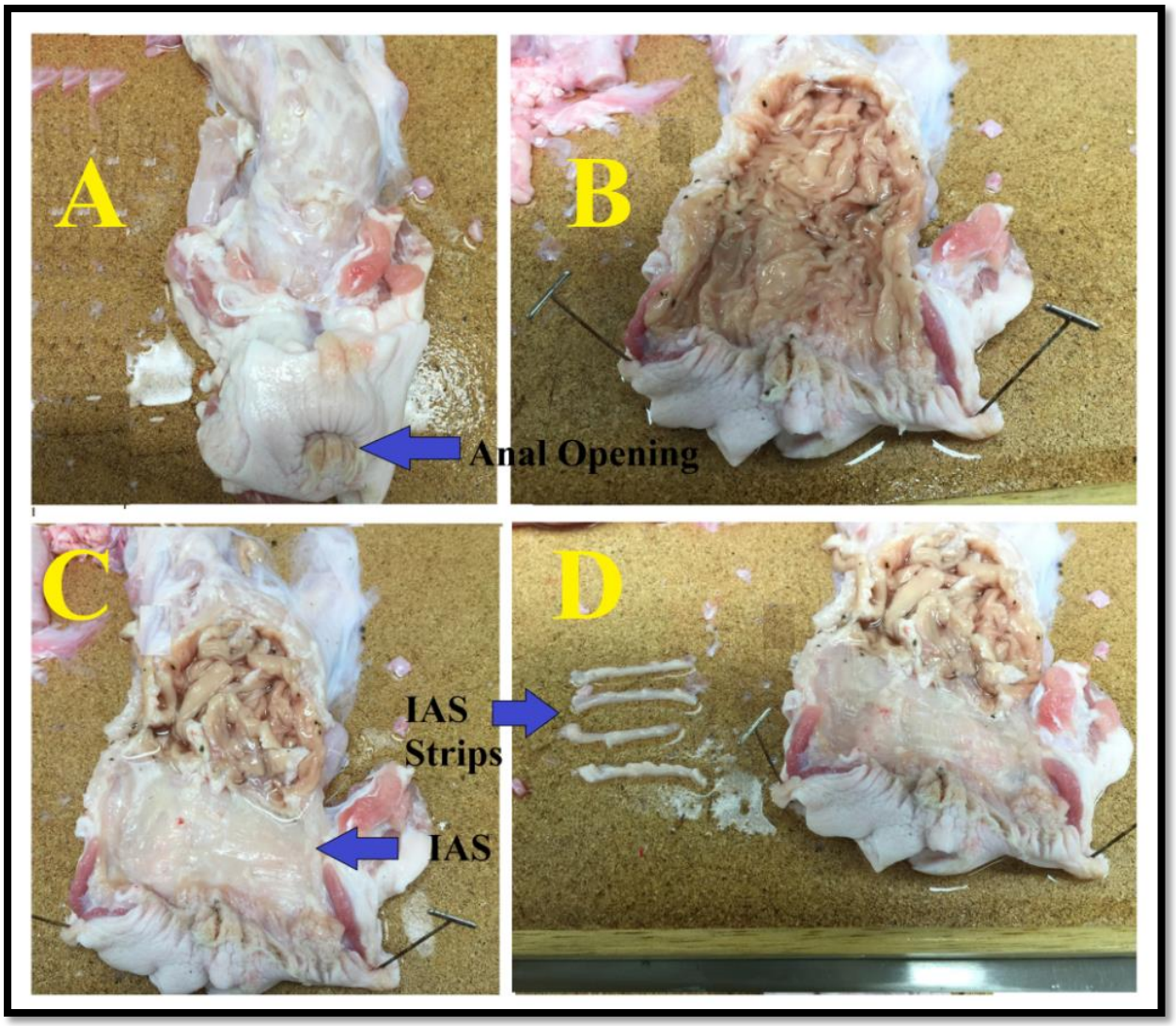


Figure 2.2 Dissection of the internal anal sphincter from the porcine anorectum. The anorectum was dissected open (B), the mucosa removed (C) and the IAS strips removed (D).

For noradrenaline responses, two concentration-curves were constructed to compare the noradrenaline response in the presence or absence of inhibitors. The initial cumulative concentration-response curves were constructed to noradrenaline followed by a 30-minutes washout. Following the washout, a second cumulative concentration-response curve to noradrenaline was obtained in all the tissues. In experiments where the effects of signalling pathways were analysed, the tissues were incubated with inhibitor of the specific pathway throughout the experiment. All experiments involving the noradrenaline curves were performed in the presence of desipramine (1μM), to block neuronal uptake-1, corticosterone (1μM), to block uptake-2 into non-neuronal tissues and propranolol (1μM) to prevent any β-AR-mediated effects.

2.6 ELECTRICAL FIELD STIMULATION (EFS)

Electrical field stimulation (EFS) is a functional in-vitro technique in which an electric current is used to stimulate the intrinsic nerves to release endogenous neurotransmitters. Internal anal sphincter tissues were placed between two platinum electrodes to study responsiveness to transmural nerve stimulation. Single square-wave pulses at supra-maximum voltage were delivered by a Grass S48 stimulator (Grass Instruments Co, MA, and U.S.A.). Internal anal sphincter tissues were set up and equilibrated in tissue baths as described and stimulated with 40volts for 1millisec duration at frequencies of 5 or 10Hz, which are the optimised parameters for stimulating neurotransmitter release in the IAS without direct stimulation of the smooth muscle (Folasire et al., 2016). Moreover, responses were abolished when tissues were stimulated in the presence of tetrodotoxin, TTX (1μM), a potent neurotoxin and selective inhibitor of neuronal sodium channel conductance.

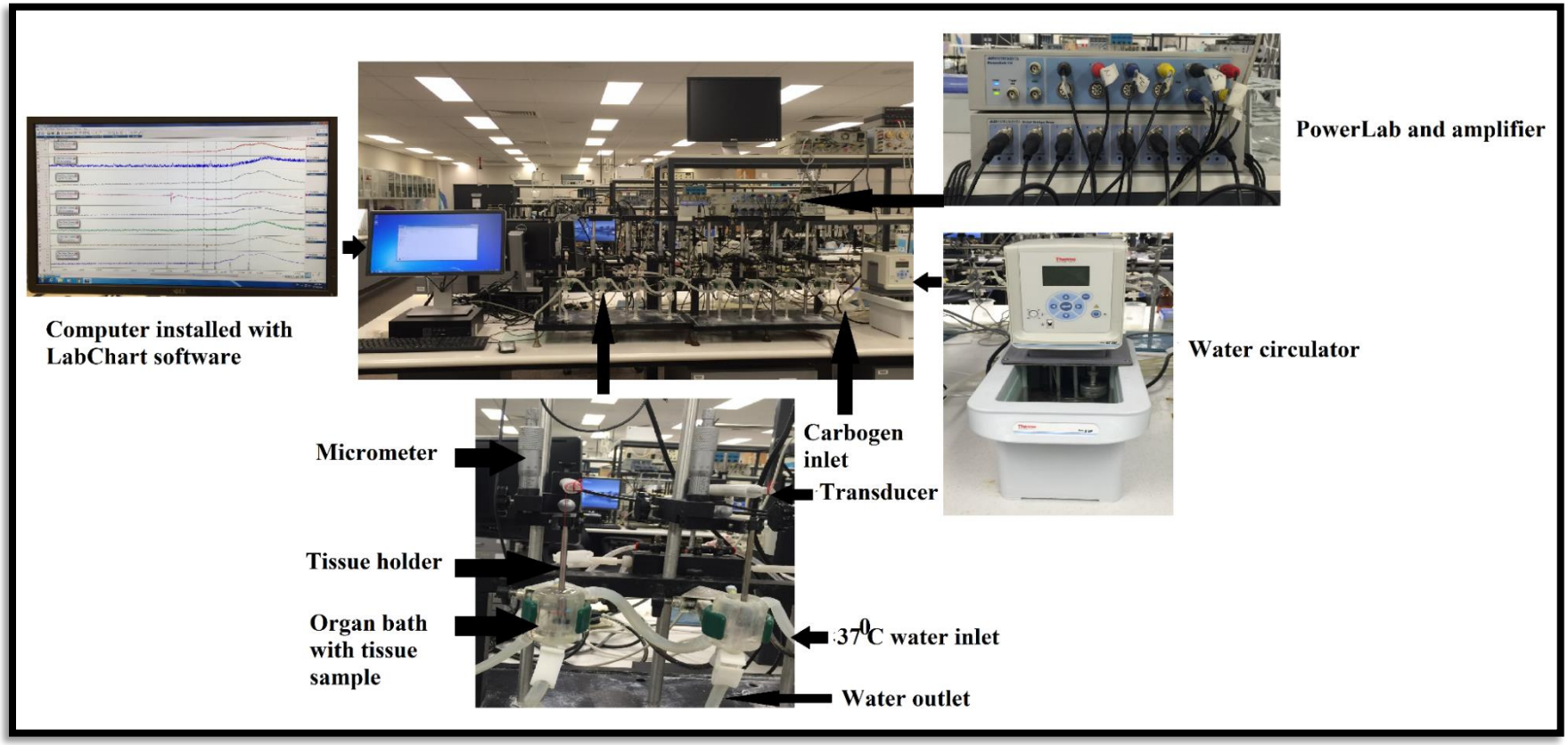


Figure 2.3 Tissue bath system.

2.7 DATA ANALYSIS AND STATISTICS

All changes in developed tension were measured and analysed using GraphPad Prism and Instat software (GraphPad, San Diego, CA). GraphPad Prism software was used for analysis of concentration-response curves via nonlinear regression to determine agonist potency (pEC50, the negative logarithm of the molar concentration that gives a half-maximal effect) and maximal responses. The agonist potencies were expressed as mean pEC_{50}±SEM and maximum responses of data were expressed as mean ± SEM. Also, in some cases EC_{50} and EC_{75} were calculated. Tissues responses normalised for weight were similar to un-normalised responses, as shown in Table 2.1 and Table 2.2. Moreover, normalisation did not have any significant effect on the agonist potency and percentage difference in maximum responses, thus un-normalised data and responses were used throughout this thesis.

For EFS experiments, changes in developed tension were represented as the percentage of the tone of the tissue at the time of stimulation. The responses seen in the presence of antagonist/ inhibitor were compared to the initial response in the absence of antagonist from the same tissue strips.

Instat software was used for statistical analysis. Where two samples were compared, unpaired or paired Student's t-test was used for analysis. Comparisons between more than two data groups were evaluated statistically with one-way ANOVA and Tukey or Bonferroni post hoc tests as appropriate. Data were considered significant only when $p<0.05$. Except otherwise indicated, all data are expressed as mean ± SEM.

	Noradrenaline		Phenylephrine		A61603	
	Young	**Old**	**Young**	**Old**	**Young**	**Old**
Max response (mN)	98.0±10.7	88.3±9.7	139.8±11.6	167.1±8.9	509.8±22.8	458.8±20.5
pEC_{50}	5.4±0.3	5.0±0.2	5.4±0.2	5.4±0.1	7.7±0.2	7.7±0.2
Percentage difference in Max (%)	9.1±18.6		19.6±12.3		10.0±9.2	
n	9	9	9	9	10	10

Table 2.1 Effect of age on urethral responses (un-normalised). Mean (±SEM) of maximum contractile responses and pEC_{50} values for agonist in the young and older porcine proximal urethral tissues. n=9-10.

	Noradrenaline		Phenylephrine		A61603	
	Young	**Old**	**Young**	**Old**	**Young**	**Old**
Max response (mN/g)	76.3±7.0	90.6±11.5	133.3±17.8	171.3±10.6	473.5±26.4	453.4±29.3
pEC_{50}	5.5±0.2	5.1±0.2	5.3±0.3	5.4±0.2	7.7±0.2	7.7±0.3
Percentage difference in Max (%)	19.6±28.5		28.5±15.2		4.2±14.7	
n	9	9	9	9	10	10

Table 2.2 Effect of age on urethral responses (normalised). Mean (±SEM) of maximum contractile responses and pEC_{50} values for agonist in the young and older porcine proximal urethral tissues. n=9-10.

2.8 DRUGS

Drugs used in this project obtained from Sigma-Aldrich (Castle Hill NSW, Australia) are listed below;

- α,β-Methylene ATP (lithium salt)
- AOAA (aminooxyacetic acid)
- Atropine (sulphate salt monohydrate)
- Carbachol (carbamoylcholine chloride)
- Corticosterone (11β,21-dihydroxyprogesterone)
- EDTA(N,N'-1,2-ethanediylbis[N-(carboxymethyl)]glycine)
- Guanethidine (1-[2-uanidinoethyl] octahydroazocine monosulfate)
- Indomethacin (1-[p-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid)
- ± Isoproterenol (1-(3′,4′-dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride)
- L- NNA (Nω-nitro-L-arginine)
- Methylene blue hydrate
- Noradrenaline (±arterenol norepinephrine [+] hydrogen tartrate)
- PAG (propargylglycine)
- PheLeu-VIP ([D-p-Cl-Phe^{6},Leu^{17}] -vasoactive intestinal peptide)
- (*R*)-(−)-Phenylephrine hydrochloride
- Propranolol (*S*)-1-isopropylamino-3-(1-naphthyloxy)-2-propanol hydrochloride)
- ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one)
- Tetrodotoxin

The following drugs listed below were obtained from Tocris Bioscience, (Ellisville, Missouri, USA).

- A61603 (N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl]methanesulfonamide hydrobromide)
- Calphostin C
- Cyclopiazonic acid
- Desipramine (10-11-dihydro-N-methyl-5H-dibenz(Z) [b,f] azepine-5-propanamine hydrochloride)

- Fasudil hydrochloride
- Nifedipine (1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester)
- Y27632 (trans-4-[(1R)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride)
- ZnPPIX (zinc protoporphyrin IX)

A comprehensive list of drugs, sources and concentrations used in this project can be found in the appendix.

CHAPTER 3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3 REGIONAL & AGE-RELATED DIFFERENCES IN THE PHARMACOLOGY OF THE URETHRA

3.1 REGIONAL DIFFERENCES IN THE LOWER URINARY TRACT

Studies of pharmacology of the urethra have yielded contradictory results that may partly be due to the use of different models, strains, and age plus the techniques used.

Regional difference within urethral structure

The structure of the urethra differs along its length, with respect to striated muscle (Karam et al., 2005b; Pel et al., 2006; Zhang et al., 2016a). In the rat, reports indicate an increase in striated muscle fibres starting from the end of the proximal urethra and peaking in the mid-urethra (Kim et al., 2007; Zhang et al., 2016a). Zhang et al., (2016a) found most of the striated muscle fibres in the middle part of the rat urethra, with the smooth muscle partly interlaced with the striated muscles. Lim et al., (2013), however, observed the striated muscle of the external urethral sphincter primarily in the proximal urethra of the rat. The circular smooth muscle is more prominent proximally in the bladder neck/proximal urethra, becoming thinner in the middle section and again increases distally in the female rat (Zhang et al., 2016a). On the other hand, longitudinal smooth muscle fibres remain thin but more consistent throughout the length of the rat urethra (Zhang et al., 2016a).

As in human (Karam et al., 2005b), the male pig urethra is composed of an outer layer of striated muscle and an inner layer of smooth muscle (Ragionieri et al., 2016). In the female pig urethra, the smooth muscle consists of an inner and outer longitudinal layer with a middle circular layer (Dass et al., 2001). Pel et al., (2006) reported striated muscle at the level of the pig bladder neck, which contrasted with the report by Dass et al., (2001), who stated that circular striated muscles are confined to the distal third of the pig urethra.

Regional differences also exist in the epithelial lining of the urethra. For example, the pig bladder neck and proximal urethra are lined with transitional urothelium (Dass et al., 2001). Several layers of epithelial cells are present at the level of the bladder neck, while fewer cell

layers have been observed in the porcine proximal urethra (Dass et al., 2001). Distally the pig urethra is lined by non-keratinising stratified squamous urothelium, and the region between consists of the pseudostratified urothelium (Dass et al., 2001). In the middle section of the rat urethra, the urothelium is surrounded by a submucosal layer containing venous plexus and smooth muscle (Zhang et al., 2016a). The vascular plexus of the pig urethra consists of predominantly longitudinally orientated blood vessels and this is most developed in the proximal urethra (Dass et al., 2001). Dass et al., (2001) suggested that the more highly developed lamina propria in the proximal urethra may reflect a larger vascular bed, and may contribute to luminal closure in this region.

In human volunteers, the maximum urethral pressure was shown to be highest in the proximal urethra during rest, cough and strain (Kirby et al., 2015). However, in the rat, the highest urethral pressure was recorded in the mid-urethra (Xu et al., 2015). Kamo et al., (2006) reported that a decreased active closure mechanism in the mid urethra mediated sneeze-induced stress urinary incontinence in a rat model of birth trauma. The mid-urethral closure mechanism was mediated by somatic nerves in rats (Kamo et al., 2006) and these nerves are important for preventing urine leakage during sneezing in rats (Kamo et al., 2003). Also, in the pig, cystometry and urethral pressure profilometry revealed a fall in mid-urethral pressure before micturition (Bridgewater et al., 1993). In the cat, during sneezing the neurally driven reflex contractions of the external urethral sphincter at the distal urethra contributed to active urethral closure mechanisms and ensured urinary continence (Bernabe et al., 2008; Julia-Guilloteau et al., 2007). In summary, the variation in the region contributing most to urethral luminal pressure seems to be species related.

Difference in the urethral regions; innervation and function

Given the differences in the structure over the length of the urethra, functional differences are expected. Whilst the sympathetic and parasympathetic nervous system innervates both the proximal urethra and bladder neck, the medial and distal urethra are to a greater extent under the control of the sympathetic innervation at least in the rabbit (Deplanne et al., 1998). In the rat, the inner longitudinal muscle layer of the proximal urethra receives an equal distribution of adrenergic and cholinergic fibres (Zhang et al., 2016a). However, in the circular layer, as well as in the outer longitudinal layer, most of the nerve endings are adrenergic. The

cholinergic fibres are located in the longitudinal and circular smooth muscle layer in the rat mid urethra (Zhang et al., 2016a). Moreover, tyrosine hydroxylase staining for adrenergic nerves was expressed more in the smooth muscle layers in the middle part of the urethra, and, the nerve distribution, identified by phalloidin and nNOS/tyrosine hydroxylase/choline acetyltransferase double staining, showed that nNOS expressing fibres were also expressed in greater quantities in the mid urethra (Zhang et al., 2016a). In the pig, the greatest density of catecholamine-, acetylcholinesterase- and peptide- positive nerves fibres was found in the smooth muscle of the distal urethra, followed by the bladder neck, middle urethra, and proximal urethra (Crowe & Burnstock, 1989).

Closure of the bladder neck and urethra depends on the adrenergic system, and the α_1-AR subtype is the predominant postjunctional α-AR in human (Brading et al., 1999) and pig (Bagot & Chess-Williams, 2006) urethral smooth muscle. The pig (Bridgewater et al., 1995) and rabbit (Larsson et al., 1986) urethra have been reported to have no regional variation in the distribution of α-ARs, with binding studies performed on membranes prepared from the rabbit bladder base, and proximal and distal regions of the urethra, revealing that the density of α_1-ARs does not significantly differ between the three areas in rabbits (Larsson et al., 1986). Similar α_1-AR densities and phenylephrine-induced contractions were recorded across all regions of the human prostatic urethra (Lepor et al., 1993). Moreover, Taki et al., (1999) also reported that noradrenaline (non-selective α-AR agonist), not clonidine (α_2-AR agonist) produced concentration-dependent contractions in all urethral regions in women, but contractions were most intense in mid to proximal urethra.

On the other hand, the density of α_2-AR is increased distally in the rabbit urethra (Larsson et al., 1986) and α_2-AR are able to mediate contractile responses in the pig distal urethral region (Bagot & Chess-Williams, 2006). Larsson et al., (1986) showed that clonidine, but not phenylephrine was more potent in the distal than the proximal region of the rabbit urethra. Thus the α_2-AR-mediated responses seem to be restricted only to the distal urethra.

The mAChR subtypes are distributed in a similar fashion throughout the length of the male rabbit urethra (Nagahama et al., 1998). Carbachol-induced contractile responses in rabbit urethra are mediated through the M_1 and/or M_3 and possibly M_2 subtypes (Mutoh et al., 1997). Carbachol produced a dose-dependent contraction of the rabbit proximal urethral region

(Mutoh et al., 1997; Nagahama et al., 1998), although, no cholinergic-mediated effect was recorded in the distal urethral region (Nagahama et al., 1998). Whilst the pig has been shown as a suitable model for understanding the lower urinary tract, α-AR agonist efficacy and potency has not been studied in detail in different regions of the urethra.

3.2 AGE-RELATED DIFFERENCES IN THE LOWER URINARY TRACT

In elderly patients, especially parous women, stress urinary incontinence is a common problem which suggests that ageing may be related to the change in contractile function in the urethra. The studies of age-related differences in the lower urinary tract have yielded contradictory results that may be partly due to a species and gender differences. Moreover, the techniques used and the ages of the models is likely to have contributed to these variations. The muscularis and lamina propria layers in the bladders of aged female rats showed significantly decreased collagen density (Lluel et al., 2000). However, Zhao et al., (2010) reported an increase in collagen content in aged male rats. Older rabbits showed significantly greater thickening of the serosa than young rabbits while young rabbits showed a significant expansion of the lamina propria (Agartan et al., 2005). Morphometric analysis has shown a significant increase in the mean thickness of the detrusor layer with age in female rats (Lluel et al., 2000). However, Zhao et al., (2010) reported a reduction in both muscle mass and urothelial thickness in aged male rats.

Age-related differences in the urethra

Carlile et al., (1988) observed an increase in the relative volume of connective tissue in aged human urethra; but no change in the smooth muscle component. The urethral length and maximum closure pressure decreased in ageing women (Trowbridge et al., 2007). Likewise, the urethral urothelium became atrophic because of a decline in oestrogen in women (Trowbridge et al., 2007). Carlile et al., (1988) also observed that ageing decreased the relative volume of striated muscle and blood vessels in the human urethra, whilst, a decline in the number and density of striated urethral sphincter nerve and muscle fibres was observed in ageing women (Pandit et al., 2000; Perucchini et al., 2002a; 2002b). Perucchini et al., (2002a)

estimated the approximate striated urethral sphincter muscle fibres lost every year to be 2% an average of 364 fibres.

Urodynamic studies consistently report a decrease in urethral closure pressures for continent women with advancing age (Rud, 1980). In a sample of nulliparous women, maximal urethral closure pressure in the senescent urethra was 40% of that in the younger urethra (Trowbridge et al., 2007). Moreover, electromyography studies showed a decrease in sphincter activity after menopause (Kenton et al., 2005). The current perception threshold was significantly higher in the distal urethra of older women, and older women experienced a loss of urethral sensation associated with loss of urethral afferent nerve function (Kenton et al., 2007a; 2007b).

There is limited literature on age-related differences in the urethra with respect to innervation, and receptor expression but, it has been shown that the intramuscular nerve density declines with age in female urethra (Pandit et al., 2000). Older women also showed a decreased motor unit action potential (striated muscle) recruitment, corroborating that ageing reduced urethral innervation (Kenton et al., 2011). Age-related changes in the function of the proximal urethra, distribution of α_1-AR binding sites and smooth muscle densities was not observed in dog urethral tissues (Ahmed et al., 2000). Similarly, Suzuki et al., (1999) also reported that α_{1A}-ARs distribution in the canine urethral smooth muscle were not affected by age and/or parity. Electrical field stimulation produced approximately the same response in the bladder base of old and younger rats (Longhurst et al., 1992).

3.3 DESENSITISATION OF RESPONSES OF THE SMOOTH MUSCLE

Desensitisation is the loss or reduction in response to subsequent exposure to the agonist. Agonist-induced desensitisation of cellular responses to stimulation of GPCR is a widespread biological phenomenon. For example, with β-ARs, desensitisation can occur upon enhanced stimulation by endogenous neurotransmitters or hormones such as in chronic heart failure (Merlet et al., 1992). The cellular and molecular mechanisms underlying agonist-induced desensitisation have largely been explored using the β_2-AR as a model (Scola et al., 2004; Tran et al., 2007).

Desensitisation of ARs can occur at the receptor level, as well as the second messenger pathway. For example, β_3-ARs desensitisation does not primarily occur at the receptor level but largely at the level of adenylyl cyclase (Vrydag et al., 2009) and perhaps, at the level of the Gs, G_i-proteins (Michel-Reher & Michel, 2013). Also, studies using β_1-ARs and/or β_2-ARs have demonstrated receptor down-regulation at mRNA level (Engelhardt et al., 1997) and G-proteins level (Adie & Milligan, 1994).

Interestingly, the susceptibility to agonist-induced down-regulation of AR appears to differ between β-ARs subtypes with β_1-ARs typically being less affected than β_2-ARs, e.g., in the heart (Nanoff et al., 1990) and HEK-293 cells (January et al., 1998). Also, the β_3-AR was thought to be insensitive to agonist-induced desensitisation because it lacks the phosphorylation sites considered to be involved in desensitisation (Nantel et al., 1993). However, Bengtsson et al., (1996) showed that exposure of β_3-ARs to noradrenaline in mouse induced a marked transient down-regulation of adipocyte β_3-ARs mRNA. Michel-Reher & Michel, (2013) also revealed that β_3-ARs are susceptible to desensitisation in transfected HEK cells. Therefore, β_3-ARs resistant to agonist-induced desensitisation seems to be cell or tissue type dependent.

Previously, the efficacy of the agonist was shown to determine desensitisation, high-efficacy β_2-AR agonists (e.g. formoterol) causing more phosphorylation and internalisation of the receptor than low efficacy agonists in HEK-293 cells (e.g. salmeterol, partial agonist) (January et al., 1997; Moore et al., 2007). However, other studies disagree, with Rosethorne et al., (2015) recently showing that different β_2-AR agonists of different efficacy produced the same degree and rate of functional desensitisation in primary human bronchial smooth muscle cells. Thus, desensitisation may be cell type specific at least for β_2-ARs.

Desensitisation of receptor-mediated responses in smooth muscle

Desensitisation of AR, especially the β-ARs, is well studied in the smooth muscle respiratory tract, with regard to repeated use of β-AR agonist, although much less well studied in the lower urinary tract (Sears et al., 1990). Repeated exposure of guinea pig (Fogli et al., 2011), rat (Finney et al., 2001) and human (Cooper et al., 2011) tracheal smooth muscle to β-AR agonists resulted in the gradual attenuation of relaxation responses. Similarly, repeated

inhalation of β-ARs agonist caused a reduction in bronchodilation in guinea-pigs in vivo (Fogli et al., 2011). Subcutaneous administration with osmotic mini-pumps delivering the long-acting β_2-ARs agonist salmeterol was demonstrated to reduce the pulmonary β_2-ARs number in rats by 70%, without affecting the levels of β_2-ARs mRNA transcripts (Finney et al., 2001). Finney et al., (2001) reported a significant reduction in the lungs membrane β_1-ARs and β_2-ARs density from animals treated with salmeterol. The decrease in receptor density mediated an impaired salmeterol-induced signalling.

With respect to the lower urinary tract, and particularly the urethra, studies investigating desensitisation are few, and most focus on the bladder. Desensitisation occurs in the bladder of the rat (Michel, 2014). Chronic stimulation of membrane-bound M_3 mAChR has been shown to result in receptor desensitisation in mouse bladder smooth muscle strips (Cha et al., 2006). Cha et al., (2006) reported that repeated pilocarpine (non-selective mAChR agonist) injections of mouse for six days induced M_3 mAChR desensitisation. Moreover, Oh et al., (2000) have shown that a repetitive carbachol application in the neonatal and adult bladder induces an attenuation of the contractile response. However, the neonatal bladder was more resistant to desensitisation, suggesting that ageing increases desensitisation of mAChR-mediated responses.

Ruggieri & Braverman, (2006) also reported a functional desensitisation of rat bladder strips to increasing concentrations of carbachol. Moreover, Ruggieri & Braverman, (2006) indicated that the repeated exposure to cumulative carbachol altered the mechanism of contraction, possibly by suppression of M_3 mAChR-mediated signalling.

Lastly, Michel, (2014) reported that agonist-induced desensitisation was prominent for the β_2-ARs involved in rat bladder relaxation but much weaker or even absent for the β_3-ARs. Moreover, β_2-ARs agonist pre-treatment reduced contractile responses to the muscarinic agonist carbachol and the receptor-independent stimulus KCl (Michel, 2014). Clinically, one year of treatment of overactive bladder with mirabegron (β_3-ARs agonist) did not provide any evidence for attenuation of the clinical response over time (Chapple et al., 2013c), thus confirming that response to the β_3-ARs agonist may also be resistant to desensitisation in the human bladder. Failure of α-AR agonists for stress urinary incontinence may be due to desensitisation of receptor.

Aim and objective

The aim of this chapter was to investigate α_1-AR-mediated responses of the porcine urethra and compare the effect of age and urethral region. In addition, desensitisation of responses of urethral tissues to subsequent addition of α_1-AR agonists was explored.

This chapter answers the following questions:

- Do α_1-AR-mediated responses differ between regions of the porcine urethra?
- Does age affect α_1-AR-mediated responses of the porcine urethra?
- Can desensitisation of receptor-mediated responses be demonstrated in the pig urethra?

3.4 MATERIALS AND METHODS

Animals and tissues

Isolated strips of porcine urethral tissues were prepared as described in chapter 2.

Experimental protocol

Following set up and equilibration for 1 hour, cumulative concentration-response curves to phenylephrine (1nM-1mM), noradrenaline (1nM-1mM) or A61603 (0.1nM-100μM) were constructed in tissues from various urethral regions and from young (average age 6 months) and older (average age 36 months) pigs. Where a second concentration-response curve was performed, tissues were washed for 30 minutes before the second addition of agonist.

Data analysis

Data were analysed and expressed as described in chapter 2.

3.5 RESULTS

Alpha$_1$-adrenoceptor-mediated responses in urethral regions

Maximum contractile responses of strips of porcine proximal urethra to cumulative concentrations of noradrenaline, phenylephrine and A61603, were greater than those of the mid and the distal urethral regions though only significant for phenylephrine and A61603 (Table 3.1, Figure 3.1, n=6-9). The pEC50 values for noradrenaline, phenylephrine and A61603 were similar for the three regions (Table 3.1: Figure 3.1), although A61603 was significantly more potent than noradrenaline and phenylephrine.

Effect of age on α_1-adrenoceptor-mediated urethral responses

Age did not affect α_1-AR-mediated responses of the urethra. Maximum contractile responses of the urethral tissues (old pigs) to cumulative concentrations of noradrenaline, phenylephrine and A61603, were similar to the maximum contractile responses of the tissues from younger pigs (Table 3.2 and Figure 3.2). Moreover, similar pEC_{50} values for noradrenaline, phenylephrine and A61603 were recorded for the young and old pig tissues.

Repeatability of receptor-mediated responses in the urethra

Maximum responses of porcine proximal urethral tissues to noradrenaline were similar for both first and second concentration-response curves (Figure 3.3). However, with phenylephrine, A61603 and carbachol, maximum responses were significantly lower in the second curves than those of the first curves (Table 3.3 and Figure 3.3; n=5-9). The percentage desensitisation of responses to phenylephrine, A61603 and carbachol were 49.9±6.9% ($p<0.001$), 55.6±7.3% ($p<0.01$) and 52.5±12.5% ($p<0.01$) respectively. Moreover, the pEC_{50} values for noradrenaline, phenylephrine, A61603 and carbachol were similar for both the first and second curves.

	Noradrenaline			Phenylephrine			A61603		
	Proximal	**Mid**	**Distal**	**Proximal**	**Mid**	**Distal**	**Proximal**	**Mid**	**Distal**
Max response (mN)	263.8±38.7	170.0±22.0	136.8±16.1	168.0±7.8	**98.0±3.2****	**76.0±3.5 ***	548.7±40.5	**224.7±14.1***	**143.2±12.6****
pEC_{50}	5.1±0.3	5.3±0.3	5.3±0.3	5.6±0.1	5.6±0.1	5.7±0.1	7.6±0.3####	7.2±0.2####	7.5±0.3####
n	8	6	6	9	9	9	8	6	6

Table 3.1 Effect of region on urethral responses. Mean (± SEM) maximum responses and pEC_{50} values for agonists in various regions of the pig urethra (36 months). ####p<0.0001 vs respective phenylephrine-induced responses (Student's t-test). *p<0.05, **p<0.01, ***p<0.001 vs. respective proximal urethral response (One-way ANOVA with Tukey post-hoc test). (n= number of strips, maximum number for each animal was 1).

	Noradrenaline		Phenylephrine		A61603	
	Young	**Old**	**Young**	**Old**	**Young**	**Old**
Max response (mN)	201.5±22.5	197.6±25.3	139.8±11.6	167.1±8.9	509.8±22.8	458.8±20.5
pEC_{50}	5.5±0.3	5.4±0.3	5.4±0.2	5.4±0.1	7.6±0.2	7.7±0.2
Percentage difference in Max (%)	4.0±15.1		19.3±12.6		10.0±9.2	
n	15	15	9	9	10	10

Table 3.2 Effect of age on proximal urethral responses. Mean (±SEM) of maximum contractile responses and pEC_{50} values for agonist in the young and older porcine proximal urethral tissues. n=9-15.

	Noradrenaline		Phenylephrine		A61603		Carbachol	
	1st Curve	**2nd Curve**	**1st Curve**	**2nd Curve**	**1st Curve**	**2nd Curve**	**1st Curve**	**2nd Curve**
Max response (mN)	138.3±9.8	138.3±6.4	133.1±19.3	**67.7±14.9 *****	514.4±32.0	**228.4±16.0 ****	103.3±11.9	**49.7±6.5****
pEC_{50}	5.4±0.2	5.1±0.1	5.6±0.4	5.7±0.6	7.3±0.2	7.3±0.3	5.6±0.3	5.3±0.3
Percentage reduction in Max response (%)	2.2±7.2		**49.9±6.9#**		**55.6±7.3##**		**52.5±12.5##**	
n	8		6		5		9	

Table 3.3 Mean (± SEM) maximum contractile responses and pEC_{50} values for agonists in proximal urethral tissues for 36 months old pigs (Data for 1st and 2nd curves is shown). **p<0.01, ***p<0.001 vs respective 1st curve responses (Paired Student's t-test). #p<0.05, ##p<0.01 vs. noradrenaline (One-way ANOVA with Tukey post-hoc test).

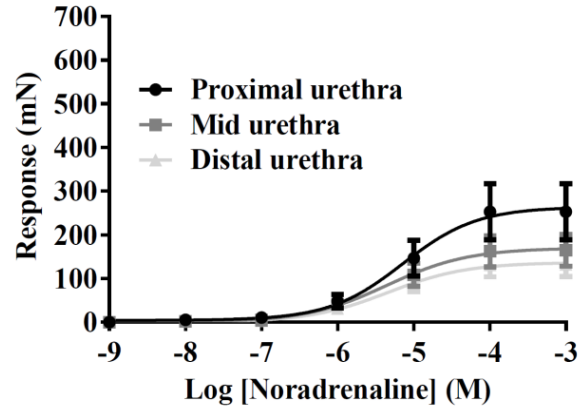
Effect of age on repeatability of concentration-response curves to phenylephrine

Repeatability of responses to cumulative concentrations of phenylephrine was not achievable in the young and old pig urethral tissues. Desensitisation of the α_1-AR-mediated responses to phenylephrine in the urethra was greater in tissues from the older pigs compared to the younger pig tissues (Table 3.4 and Figure 3.4; n=9). pEC_{50} values for phenylephrine were similar between the young and older pigs for the first and second concentration-response curves.

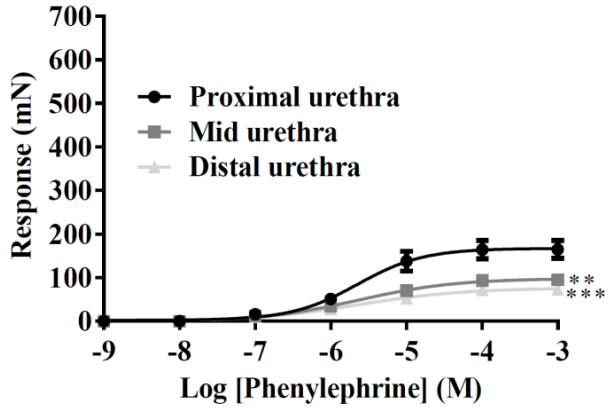
	Young		Old	
	1st Curve	**2nd Curve**	**1st Curve**	**2nd Curve**
Max response (mN)	124.8±15.3	91.7±19.6	163.7±13.5	**54.6±8.2*** **
pEC_{50}	5.4±0.3	5.4±0.5	5.7±0.2	5.7±0.4
Percentage reduction in Max response (%)	29.6±19.4		67.2±5.8	
N	9		9	

Table 3.4 Mean (± SEM) maximum contractile responses and pEC_{50} values for phenylephrine in young and old porcine urethral tissues (Data for first and the second curve is shown). ***$p<0.001$ vs 1st curve maximum (Paired Student's t-test).

Noradrenaline



Phenylephrine



A61603

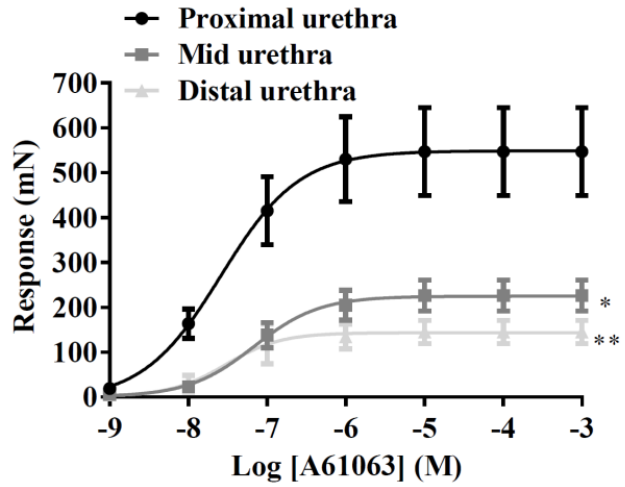
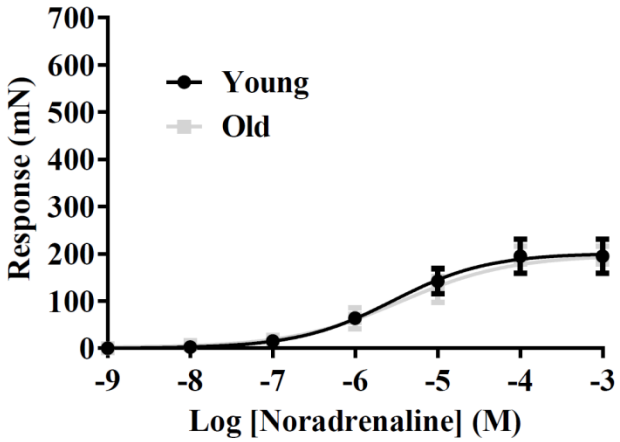
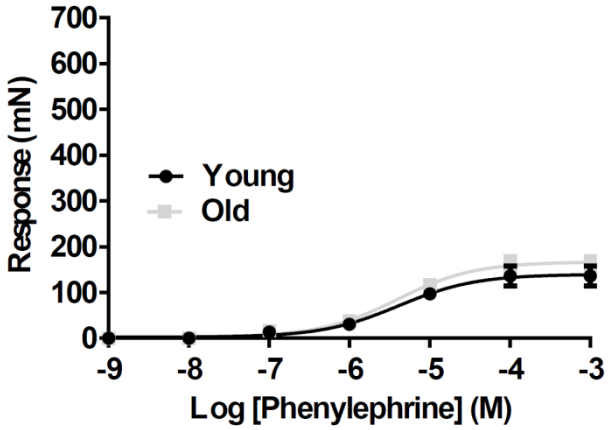


Figure 3.1 Mean concentration-response curves to agonists on various regions of the pig urethra from 36 months old animals; n=6-9. *p<0.05, **p<0.01, ***p<0.001 vs. maximum responses of proximal urethra (One-way ANOVA with Tukey post-hoc test).

Noradrenaline



Phenylephrine



A61603

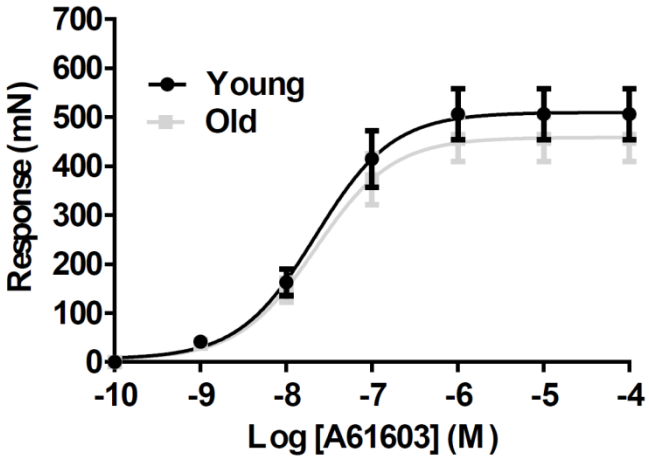


Figure 3.2 Comparison of the mean concentration-response curves to agonists in proximal urethral tissues from old and younger pigs; n=9-15.

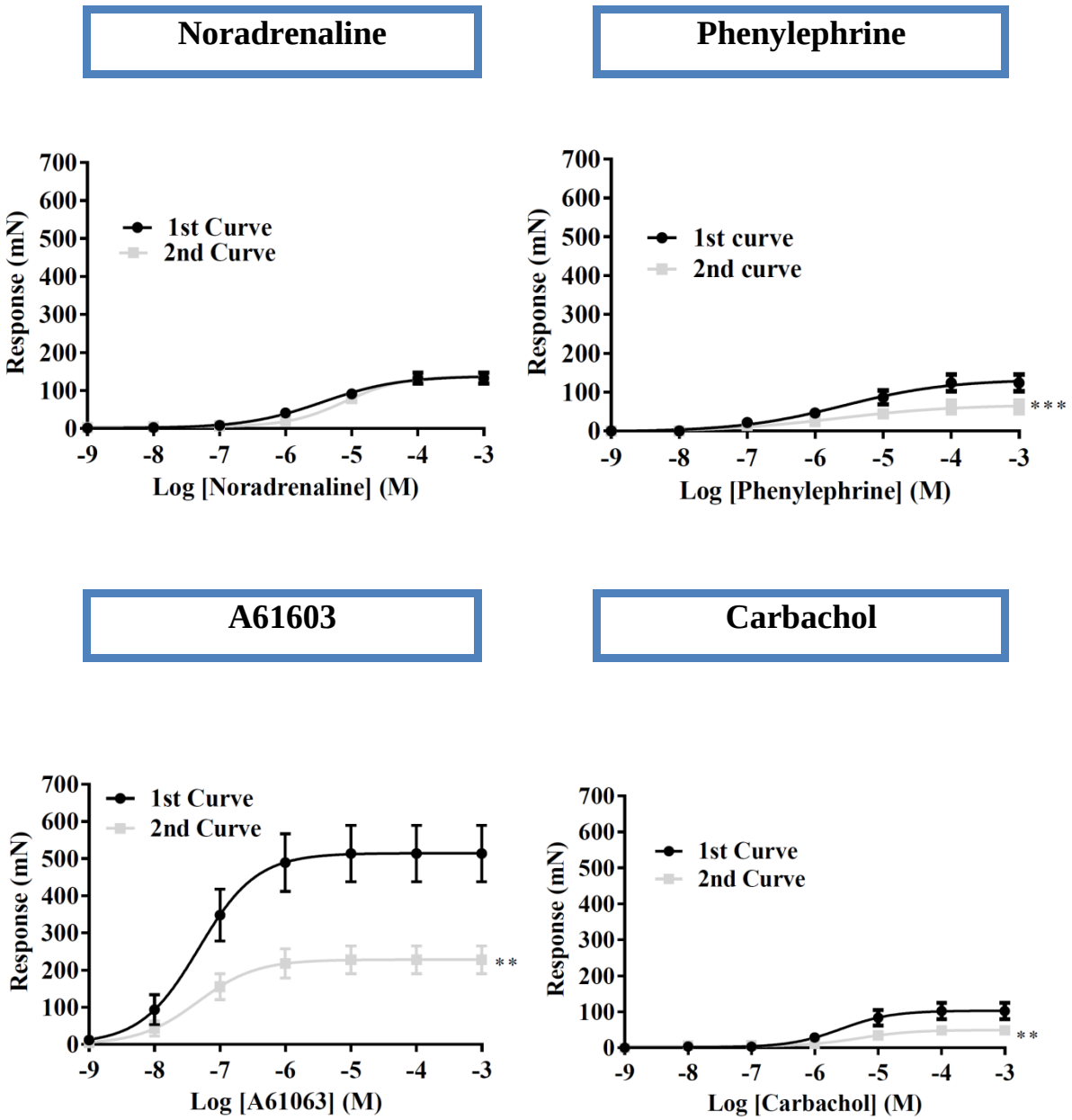


Figure 3.3 Comparison of the 1st and 2nd mean concentration-response curves of proximal urethral tissues to agonists (36 months old pigs); n=5-9. **p<0.01 and ***p<0.001 vs 1st curve responses (Paired Student's t-test).

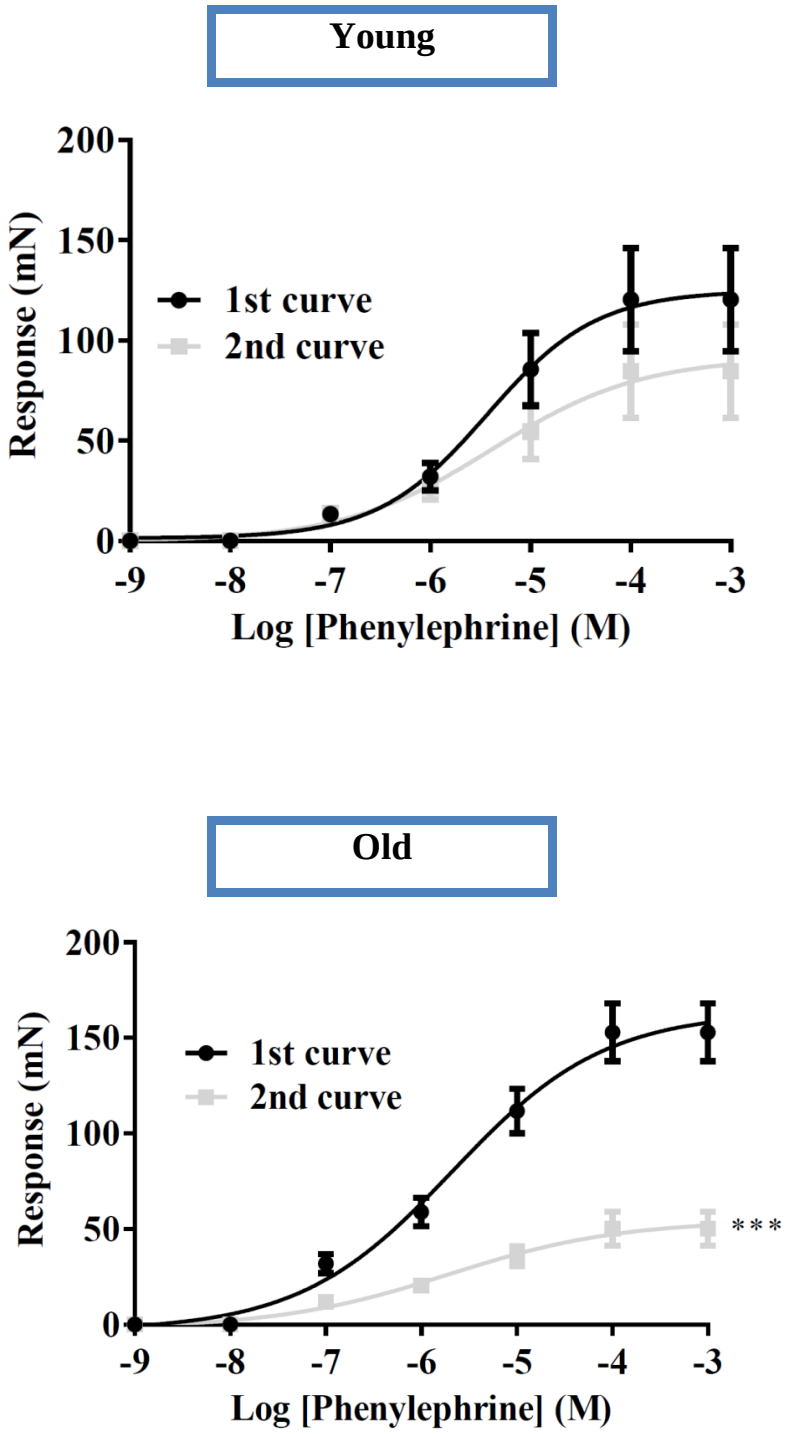


Figure 3.4 Concentration-response curves to phenylephrine in proximal urethral tissues. Results were expressed as mean ± SEM; n=9. ***p<0.001 vs. first curve response (Paired Student's t-test).

3.6 DISCUSSION

Do α_1-adrenoceptor-mediated responses differ between regions of the porcine urethra?

For urinary continence, the resting luminal pressure of the urethra must be maintained to prevent urine leakage. However, the urethral region with the greatest contribution to resting pressure is still a matter for debate. Responses of urethral tissues to phenylephrine and other α_1-AR agonists, in-vivo, in human and in animal models have shown region and species-dependent variations along the length of the urethra (Conlon et al., 2009; Walters et al., 2006; Zhang et al., 2016a). For example, in the rat the greatest responses to phenylephrine were found in the proximal urethra, whilst in the guinea-pig, the opposite result has been shown, with the greatest amplitude of contraction seen at the distal end of the urethra (Walters et al., 2006). Clinically, urodynamic studies suggest a significant contribution of the proximal urethra in maintaining continence (Bagi, 2002; Bagi et al., 2002). Moreover, Kirby et al., (2015) reported highest maximum urethral closure pressures in the proximal urethra during different manoeuvres, including rest, cough and strain with high-resolution manometry catheter. Using urethral pressure reflectometry, Aagaard et al., (2014) also reported an increase in the urethral opening and closing pressure and hysteresis from the bladder neck to the sphincter region (proximal urethra) in men. Together, these studies suggested that the functional responses along the urethra may vary between species.

In this project, we aimed to establish any regional differences in α_1-AR-mediated contractile response in the pig and determine the similarity to human urethra. Maximum contractile responses to noradrenaline, phenylephrine and A61603 were greatest in the proximal region of the porcine urethra, followed by the mid and then distal urethral regions. Cystometry reports from studies using the porcine model have also described the proximal urethra as the high-pressure zone with high contractile tone (Greenland et al., 1996). The high-pressure zone, approximately 3cm below the bladder neck (Werkstrom et al., 1995), correlates with the proximal region used in this thesis as the region with greatest agonist-induced contractile responses. This finding supports earlier urethral pressure profilometry and cytometry observations in human (Aagaard et al., 2014; Kirby et al., 2015; Taki et al., 1999).

The novelty in the present experiment was in the elucidation of α_1-AR-mediated responses in the porcine urethral regions using agonists of different efficacy and potency. A61603 and phenylephrine were used in addition to noradrenaline, and the responses to the three agonists were consistently greater in the proximal urethral region though only significant for phenylephrine and A61603. A close look at Greenland et al., (1996) report showed that they used younger pigs (60 to 100kg) in their experiments. In this study, we used much older pigs ($\geqslant$180kg). Therefore, we have shown that the proximal urethral region is associated with greatest agonist-induced contractile responses in the older pig which is similar to the data from young pig reported by Greenland et al., (1996).

A61603 caused a greater contractile response than the other three agonists, and was the most potent of all the agonists in urethral tissues. These results are consistent with a large body of evidence stretching back decades showing that A61603 is highly potent and specific for α_{1A}-ARs, in the urethra (Bagot & Chess-Williams, 2006) and other tissues including rat prostate (Thiyagarajan et al., 2002), dog prostate, rat vas deferens (Knepper et al., 1995), rabbit arteries (Smith et al., 1997), rat spleen (Zhou & Vargas, 1996) and dog arteries (Argyle & McGrath, 2000). Phenylephrine and noradrenaline had similar potency in this experiment, which is also reflected in the literature (Bagot & Chess-Williams, 2006; Minneman et al., 1994).

Does age affect α_1-adrenoceptor-mediated responses in the porcine urethra?

Age may change the morphology (Agartan et al., 2005; Zhao et al., 2010) and functional responses (Daly et al., 2014; Tykocki et al., 2013) of the lower urinary tract. The research on the effect of ageing on lower urinary tract morphology and functional characteristics has provided contradictory results. Detrusor contractility, bladder sensation, and urethral pressure decline with ageing in women (Pfisterer et al., 2006). However, other reports have shown that ageing does not affect these factors (Ameda et al., 1999; Gosling, 1997). These variations can be related to animal species or strain used, sex or experimental procedure.

There is convincing evidence showing that the urethra expresses α_1-ARs (Nasu et al., 1998; Yono et al., 2004). Specifically, the α_{1A}-AR subtype is the main α_1-AR present in the urethra of different animal species, including man (Nasu et al., 1998; Testa et al., 1993; Walden et al.,

1997). There is also evidence that urethral tissue contraction occurs predominantly if not exclusively, via α_1-ARs (Bagot & Chess-Williams, 2006; Chess-Williams et al., 1994; Kedia et al., 2013). Therefore, changes in α_1-AR-mediated responses could have a negative effect on continence, although, it is not yet clear how α_1-AR-mediated responses are affected by ageing. In this study, we sought to examine how ageing affects α_1-AR-mediated responses of the urethral tissues.

Contractions of urethral tissues to cumulative concentrations of noradrenaline, phenylephrine and A61603 were found to be similar in the young and older porcine urethral tissues. Moreover, potencies of the agonists were similar between the young and older porcine urethral tissues. These results suggest that ageing does not affect α_1-AR-mediated contractile responses in the pig. The results presented here were consistent with those reported in the literature, although in different species. There was no significant age-dependent difference in the total α_1-AR density and mRNA expression of all three α_1-AR subtypes or the maximum contractile responses to phenylephrine in the bladder base of rat (Yono et al., 2006). Furthermore, receptor-mediated responses were similar in young and older dog urethral tissues (Suzuki et al., 1999), and no correlation could be established between age and urethral pressure in healthy males (Bagi et al., 2002). In the aged human prostate, the contractile response to exogenously administered α_1-AR agonists also remains unchanged with ageing (Tsujii et al., 1992). Moreover, the amplitude of urethral responses during sneezing was unchanged in middle-aged rats compared to younger rats (Kitta et al., 2016). Similar α_1-AR-mediated responses were recorded in young and older urethral tissues in the present study, could be associated with the findings of unchanged receptor expression in the urethra, as shown in the dogs and rats (Ahmed et al., 2000; Suzuki et al., 1999; Yono et al., 2006).

The age of young pigs used in this study correlate to adolescence in human, whilst the older pigs correlate to 60 years and above. Thus from this study, ageing does not appear to affect the α_1-AR-response.

Can desensitisation of receptor-mediated responses be demonstrated in pig urethra?

Alpha$_1$-AR agonists have been reported to increase urethral luminal pressure and improve stress urinary incontinence in animal models (Blue et al., 2004; Segev et al., 2015) and human (Collste & Lindskog, 1987; Musselman et al., 2004; Radley et al., 2001). Alpha$_1$-AR agonists have also shown efficacy in reducing stress urinary incontinence symptoms in clinical studies (Radley et al., 2001; Segev et al., 2015). However, they are associated with severe cardiovascular effects (Dave et al., 2002; O'Neill et al., 2001; Radley et al., 2001). Also, the effect on maximum urethral closure pressure improvement was fairly minimal in beagle dogs (Noel et al., 2010; 2013). Desensitisation of the α-ARs has been proposed by several authors to explain this observation (Akinaga et al., 2013). Indeed desensitisation of ARs has been reported for β-ARs in asthma treatment (Lin et al., 2012) and α_1-ARs in HEK-293 cells (Castillo-Badillo et al., 2015). Thus, the efficacy of treatment with agonists at α_1-AR subtypes over time may be limited by agonist-induced (homologous) desensitisation and/or by unrelated stimuli (heterologous) desensitisation (Castillo-Badillo et al., 2015). Functional desensitisation of α_1-ARs occurs in a tissue- and cell type-dependent manner and as far as we are aware, this work is the first to investigate desensitisation of α_1-AR-mediated responses in the porcine urethra, with agonists of different efficacy and potency.

This study showed desensitisation of subsequent contractile responses of urethral tissues to phenylephrine and A61603. The desensitisation produced was greatest for A61603 (55.6%), followed by phenylephrine (49.9%). However, noradrenaline responses did not desensitise upon repeated application. All tissues were washed for the same amount of time, to return tissues to baseline tension, thus differential washing cannot explain the desensitisation of phenylephrine and A61603-induced responses. A61603 is a full agonist with a maximum response and potency significantly greater than that of noradrenaline, whilst phenylephrine and noradrenaline have similar efficacy and potency (Bagot & Chess-Williams, 2006). Thus, noradrenaline the endogenous agonist for these receptors, was actually a partial agonist compared to A61603. This is surprising but not unexpected since other studies have also reported the greater maximum responses to A61603 (Knepper et al., 1995). Thus, the agonist with the greatest efficacy, potency and affinity for α_{1A}-ARs induced greatest desensitisation.

In addition, the desensitisation may be due to fall in receptor number, however, ascertaining this hypothesis is beyond the scope of this project.

Most studies on desensitisation of AR have been carried out on the β-AR subtype, and in cell studies. Drawing parallels from these studies, a similar effect to the present study has been shown for the β-ARs in mast cells, with desensitisation of subsequent responses following exposure to β-AR agonists (Chong et al., 2003; Scola et al., 2004). Similarly, the efficacies of different agonists were correlated to the degree of desensitisation of the β_2-ARs expressed in HEK-293 cell lines (January et al., 1997; Pittman et al., 1984) and μ-opioid receptors expressed in AtT20 cells (Borgland et al., 2003) or HEK-293 cell lines (McPherson et al., 2010). Benovic et al., (1988) established an excellent correlation between the ability of β-AR agonists to stimulate adenylate cyclase activity and receptor phosphorylation by GRKs. Also, McPherson et al., (2010) established an excellent correlation between efficacy for G-protein activation and arrestin recruitment for μ-opioid receptors. The intensity of the peptide maps of receptor phosphorylated by GRK in the presence of partial agonists was lower compared to full agonists (Benovic et al., 1988). High-efficacy β_2-AR agonists (e.g. formoterol) caused more phosphorylation and internalisation of the receptor than low efficacy agonists (e.g. salmeterol) in HEK-293 cell lines (January et al., 1998). Also, salmeterol binding to β-ARs induced an active receptor state that was unable to recruit arrestin or undergoes significant endocytosis or degradation despite stimulating considerable GRK-site phosphorylation (Moore et al., 2007). These reports suggest that high-efficacy agonists mediate greater desensitisation because they induce a greater intracellular signalling, receptor phosphorylation and internalisation (Benovic et al., 1988; McPherson et al., 2010; Scola et al., 2004). Greater efficacy would also result in a greater number of activated receptors than for partial agonists (Benovic et al., 1988; McPherson et al., 2010).

In contrast, earlier studies using equi-efficient concentration of β-AR agonists in human airway cells showed that desensitisation induced by agonists were independent of the agonist efficacies (Duringer et al., 2009). Duringer et al., (2009) used formoterol which is a full agonist and salmeterol which is a partial agonist at β-ARs (Condemi, 2001; Palmqvist et al., 1999). Using equi-efficient concentrations of formoterol and salmeterol, formoterol induced the least reduction of responsiveness (cyclic AMP response) after the 1 hour prior exposure in human airway cells (Duringer et al., 2009). Their study showed that under conditions of

continuous agonist stimulation, reductions in receptor responsiveness were relative to the initial functional effect and similar for all agonists studied. Duringer et al., (2009) used equi-efficient concentrations of agonist rather than agonist occupancy in their experiments. Using equi-effective concentrations, full agonist would occupy fewer receptors to give the response reported by Duringer et al., (2009). Thus, the less desensitisation induced by formoterol may be related to the number of receptors occupied i.e. fewer thus, a small desensitisation.

In the present study, A61603 was more potent than noradrenaline and phenylephrine at the α_{1A}-AR subtype of the pig urethra confirming full agonist activity at α_{1A}-ARs (Yoshiki et al., 2013). Potency of each agonist examined was also proportional to the desensitisation produced (noradrenaline exempted). The ability of phenylephrine and A61603 to induce desensitisation could be mediated by GRK or PKC associated phosphorylation and modulation of desensitisation (Xiang et al., 2001). Desensitisation mediated by PKC has been shown to be induced by A61603 in HEK-293 cells (Xiang et al., 2001) and rat cardiomyocytes (Rohde et al., 2000), and by phenylephrine in the rat aorta (Zhao et al., 2015). However, noradrenaline may be a weak activator of PKC in the urethra or mediates down-regulation of PKC, as shown in the rat cardiomyocytes (Rohde et al., 2000), which may explain the lack of desensitisation observed with noradrenaline. Moreover, total repeatability of noradrenaline-mediated responses in the present study after the washing period also suggested a complete recovery of surface receptors from intracellular pools, as shown in the Rat-1 fibroblasts (Morris et al., 2004). A61603, which has a higher affinity for α_{1A}-ARs, compared to phenylephrine and noradrenaline (Knepper et al., 1995; Thiyagarajan et al., 2002) and a higher affinity for α-ARs has been associated with greater phosphoinositide hydrolysis (Knepper et al., 1995). Moreover, diacylglycerol a product of phosphoinositide hydrolysis, induces Ca^{2+} influx and PKC activation, which can also mediate desensitisation (Xiang et al., 2001). Therefore, the greater desensitisation seen with A61603 may also be proportional to its greater ability to induce phosphoinositide hydrolysis and inositol 1, 4, 5-tris-phosphate/diacylglycerol production, resulting in PKC-mediated desensitisation. Intracellular pathway involved in desensitisation is further investigated in chapter 6.

Muscarinic acetylcholine receptor activation is also known to contract the proximal urethra/bladder neck (Nagahama et al., 1998; Taki et al., 1999; Yamanishi et al., 2002a). Therefore, we investigated desensitisation of carbachol-mediated responses in the urethra.

The potency of carbachol was lower than that of A61603, phenylephrine and noradrenaline, confirming that the α_1-ARs mediate the predominant contractile responses of the urethra (Bagot & Chess-Williams, 2006). Desensitisation of muscarinic responses by prior application of carbachol (52.5%) was similar to the desensitisation of α_1-AR responses produced by A61603 and phenylephrine. We believe this report is the first to show desensitisation of mAChR-mediated responses to carbachol in porcine urethral tissues. Desensitisation of the mAChRs has been reported in the mouse bladder (Cha et al., 2006). Desensitisation of all mAChRs (except M_1) has been shown to be mediated by the GRK2 (Tsuga et al., 1998), whilst, M_1 mAChR internalisation is dependent on arrestin, G protein and the third intracellular loop of these receptors, as shown in CHO cells (Tsuga et al., 1998; Yeatman et al., 2014). Budd et al., (1999) also showed that M_3-mAChR mediated heterologous phosphorylation of β_2-ARs in a GRK-independent fashion, but via PKC. Therefore, there is a possibility of the desensitisation of subsequent responses to carbachol being induced by GRK/ PKC (Borroto-Escuela et al., 2011; Hosey et al., 1995), arrestin and G-protein recruitment (Tsuga et al., 1998; Yeatman et al., 2014).

The extent and rate of internalisation of M_1-M_5 mAChR expressed in COS-7 and BHK-21 cells has been shown to differ from one receptor subtype to another (Tsuga et al., 1998). Specifically, M_2 mAChRs were not re-sensitised in most cases and did not recycle back to the plasma membrane in HEK-293 cells compared to M_1 mAChR (Vogler et al., 1998). Also, the dominant negative arrestin mutant only displayed a significant effect on M_1, M_3 and M_4 mAChR sequestration, which indicated that the receptor subtypes may differ in their requirement for arrestin (Vogler et al., 1999). Coincidentally, the M_2 and M_3 mAChR subtypes which were susceptible to desensitisation (Vogler et al., 1998; 1999) are the receptors that mediate carbachol-induced contraction of the porcine urethral smooth muscle (Yamanishi et al., 2002a; 2002d). Therefore, these results suggest that the desensitisation of responses to carbachol involving M_2 and M_3 receptors in the porcine urethra. However, a thorough study researching the receptor protein density and intracellular signalling is needed to confirm the contribution of these receptors to desensitisation, and precise intracellular mechanisms.

Does age affect desensitisation of α_1-adrenoceptor-mediated responses?

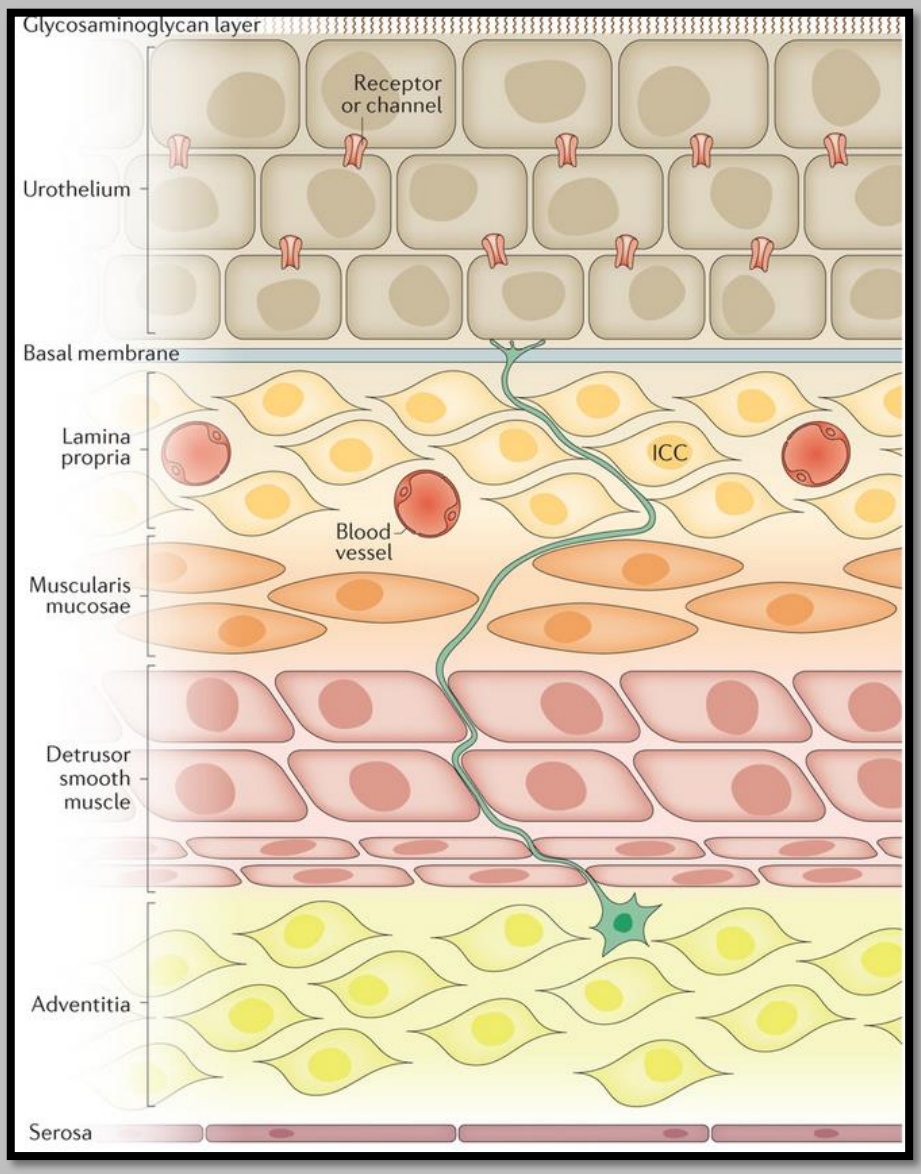
Alpha$_1$-ARs are prospective drug targets for stress urinary incontinence, however, as shown above, the efficacy of treatment with α_{1A}-AR agonists may be limited by desensitisation. The effect of desensitisation may increase or decrease with ageing; a phenomenon is already shown in the vascular system (Dinenno et al., 1999; 2001; Smith et al., 2007). Therefore, we investigated the effect of age on desensitisation of contractile responses of the urethral tissues to phenylephrine. We found that α_1-AR-mediated responses are not altered with age in porcine urethral tissues in this chapter and when considering the downstream effect of ageing, no differences in PKCα activity were reported between young and older mice, although in the trachea and lungs (Bailey et al., 2014). Other researchers have also shown no change in PKC isoforms in the ageing brain (Battaini & Pascale, 2005), the ageing myocardium (Korzick et al., 2001), and in lymphocytes (Whisler & Grants, 1993), and no change in GRK activity in the ageing human heart (Leineweber et al., 2003). Thus, we hypothesised that desensitisation of contractile response of the urethral tissues to phenylephrine would be age-independent. However, this was not the case and desensitisation of responses to phenylephrine was in fact greater in the old compared to the younger urethral tissues. Jiang et al., (1993) showed that age-related desensitisation was related to decrements in β-AR-mediated cyclic AMP accumulation, phosphorylation of phospholamban and troponin I. Moreover, an age-related increase in GRK (Iaccarino et al., 2005; Ping et al., 1997; Rengo 2014), and increase in G_i (Fu et al., 1992) were reported in human heart, and an age-related increase in β-arrestin, GRK2 and GRK5 expression have been reported in the rat (Lymperopoulos et al., 2007; Rengo 2012a; 2012b; 2012c; Vinge et al., 2001). The increased desensitisation of subsequent contractile response to phenylephrine in the older porcine urethra may therefore involve several possible intracellular mechanisms including increases in Gαq, GRK, PKC and β-arrestin expression. Greater desensitisation in the older pig tissue may also be associated with an increase in Ca^{2+} influx and increase in Ca^{2+} release from the sarcoplasmic reticulum, as found by Lopes et al., (2006) in intestinal smooth muscle. The specific signalling pathways contributing to desensitisation of subsequent contractile responses in the urethral tissues is investigated further in chapter 6.

Conclusion

Greatest α_1-AR-mediated responses were recorded in the proximal porcine urethral region. Phenylephrine, A61603 and carbachol responses showed desensitisation upon repeated application. However, responses to noradrenaline did not desensitise. Moreover, age had no significant effect on α_1-AR-mediated responses, but responses to phenylephrine desensitised to a greater extent in the older urethral tissues.

Based on the results from this chapter, the proximal urethra was used for the rest of the studies in this thesis, except where stated. Moreover, repetition of concentration-response curves to phenylephrine, A61603 and carbachol were avoided for the rest of the experimental work, to prevent desensitisation interfering with results. Since the α-AR-mediated responses were similar for the young and old urethral tissues, porcine urethral tissues from older animals were used for the rest of the studies in this thesis except, were specified.

CHAPTER 4



Modified from Merrill et al., 2016.

4 THE EFFECT OF THE UROTHELIUM ON URETHRAL RESPONSES

4.1 UROTHELIUM DERIVED INHIBITORY FACTOR

In the 80's, Furchgott & Zawadzki, (1980) observed that in a rabbit aorta ring with endothelium preparation acetylcholine produced marked relaxation. They showed that relaxation of isolated preparations of rabbit aorta and other blood vessels by acetylcholine requires the presence of endothelial cells, and that acetylcholine, acting on mAChR of these cells, stimulates the release of a substance(s) that causes relaxation of the vascular smooth muscle. They proposed that this inhibitory effect may be one of the principal mechanisms for acetylcholine-induced vasodilation in vivo (Furchgott & Zawadzki, 1980).

Maggi et al., (1987) was one of the first to report that contractile responses to the sensory nerve mediator substance P in the guinea-pig urinary bladder were smaller when the urothelium was intact. This suggested the release of an inhibitory factor from the guinea-pig urothelium. Also, removal of the mucosa enhanced responses to transmural nerve stimulation at low voltages and pulse durations in guinea-pig bladder (Krasnopolsky et al., 1995).

Levin and colleagues (1995) found that the urothelium had a significant inhibitory effect on the contractile response of the cat bladder to stimulation with several agonists. Later, Hawthorn et al. (2000) reported an enhanced response to carbachol in tissues without urothelium in the pig urinary bladder. When a second urothelium-intact tissue was co-incubated, the responses returned to a lower amplitude which was due to the effect of the urothelium suggesting the release of a diffusable inhibitory agent (Hawthorn et al., 2000). Furthermore, the presence of a second bladder strip without urothelium, failed to inhibit contractions in the first strip, suggesting the inhibitory factor is released by the urothelium and not the detrusor smooth muscle. Hawthorn et al. (2000) conclusion was supported further by the finding that responses to carbachol were potentiated following removal of the urothelium from previously intact bladder strips. Similar to the work of Fovaeus et al., (1999) in the rat bladder, Hawthorn et al. (2000) showed that NO does not appear to mediate the effect of the pig bladder urothelium, since L-NOARG (NOS inhibitor) had no effect on the inhibition. Moreover, inhibition of guanylate cyclase, and incubation with indomethacin,

suramin, propranolol, and potassium channel blocker TEA had no effect on the urothelium induced inhibition, excluding cyclooxygenase products, P2Y receptors, β-ARs, gamma-aminobutyric acid and voltage-activated or ATP-dependent K^+ channels respectively from potential factors mediating the effect. Templeman et al., (2002) shared a similar inhibitory effect of the urothelium in the pig trigone. Contractile responses to carbachol and histamine but not to phenylephrine were depressed in the presence of the urothelium. However, activation of mAChRs and histamine receptors resulted in the responses of intact urothelium strips to phenylephrine being significantly depressed. Thus, Templeman et al., (2002) showed that the urothelium derived factor appears to mediate cross-talk between the mAChR, histamine receptor and the α-AR in the pig trigone.

Beta-AR agonists, possibly via urothelial β_3-ARs (Masunaga et al. 2010) also seem to stimulate the release of this unidentified factor which inhibits detrusor contractility directly or indirectly (Murakami et al. 2007). Urothelium-derived inhibitory factor appears to be released in the human bladder, as responses to EFS or carbachol were also enhanced in the absence of urothelium (Chaiyaprasithi et al., 2003). However, contractions elicited by KCl and by neurokinin A were not modified by the urothelium. In the human bladder, the urothelium derived inhibitory factor was not NO, cyclooxygenase product and not mediated by guanylate cyclase and β-ARs (Chaiyaprasithi et al., 2003).

Although studies have indicated that the urothelium-derived inhibitory factor is diffusible within an organ bath (Fovaeus et al., 1998; 1999; Hawthorn et al. 2000; Templeman et al., 2002; Chaiyaprasithi et al., 2003) and over a longer distance (from one bath to another) (Guan et al., 2014a), the chemical nature of this 'urothelium-derived relaxing factor' has not been resolved. Also, most studies on the urothelium are based in the bladder and less is known about the physiology and pharmacology of the urethral urothelium. Particularly it is not yet known whether the urethral urothelium affects the underlying smooth muscle response. Since the maintenance of luminal pressure of the urethra is paramount to maintaining continence, it is important to know whether the urothelium is involved in modulating the porcine urethral responses, tone and luminal pressure. In addition, dysfunction of any urothelial mechanism may be involved in the pathology of disorders such as stress urinary incontinence, and provide a new potential target for therapeutic intervention.

Study aim

The aim of this chapter was to investigate the role of the urothelium in the porcine urethra. Specifically, this study focussed on:

- Does the urothelium affect α_1-AR, β-AR and mAChR-mediated responses of the urethra?
- Does the effect of the urothelium depend on age or urethral region?
- What factors contribute to any effect?
- Does the urothelium affect desensitisation of α_1-AR-mediated responses?

4.2 MATERIALS AND METHODS

Methods

To determine the effect of the urothelium/lamina propria (LP) on receptor-mediated responses, the urothelium/LP was carefully dissected off and separated from the smooth muscle of the urethra. The tissues with and without urothelium/LP were set up in pairs, in 8ml organ baths, as described in chapter 2. Following set up and equilibration for 1 hour, cumulative concentration-response curves to agonists (noradrenaline, phenylephrine, A61603, carbachol and isoprenaline) were constructed.

To investigate what factors may be contributing to the effect of urothelium/LP, urethral tissue strips with or without urothelium/LP were set up in pairs and incubated with inhibitors for 30 minutes after equilibration. The tissues were then contracted to cumulative concentrations of agonists and responses compared with control tissues (absence of inhibitors). Indomethacin (10μM) and LNNA (100μM) were used to block prostaglandins and NO production respectively. Concentration-response data were plotted and analysed as described in chapter 2.

4.3 RESULTS

Investigation of α_1-adrenoceptor-mediated responses in urethral tissues

As shown in an earlier chapter, A61603 produced significantly greater contractions than the endogenous agonist noradrenaline, or phenylephrine (Table 4.1; Figure 4.1). Also, the pEC_{50} values for A61603 were significantly greater than for the other agonists, showing greater potency (Table 4.1).

	Noradrenaline	Phenylephrine	A61603
Maximum response (mN)	148.3±8.9	121.4±11.1	**339.3±23.2****
pEC_{50}	5.0±0.1	5.5±0.2	**7.7±0.3*****
n	4	6	10

Table 4.1 Mean (±SEM) maximum contractile response and pEC_{50} values for agonists for contractile responses of porcine urethral tissues to noradrenaline, phenylephrine and A61603. **p<0.01, ***p<0.001 vs. response to noradrenaline (one way ANOVA, with Tukey post hoc test).

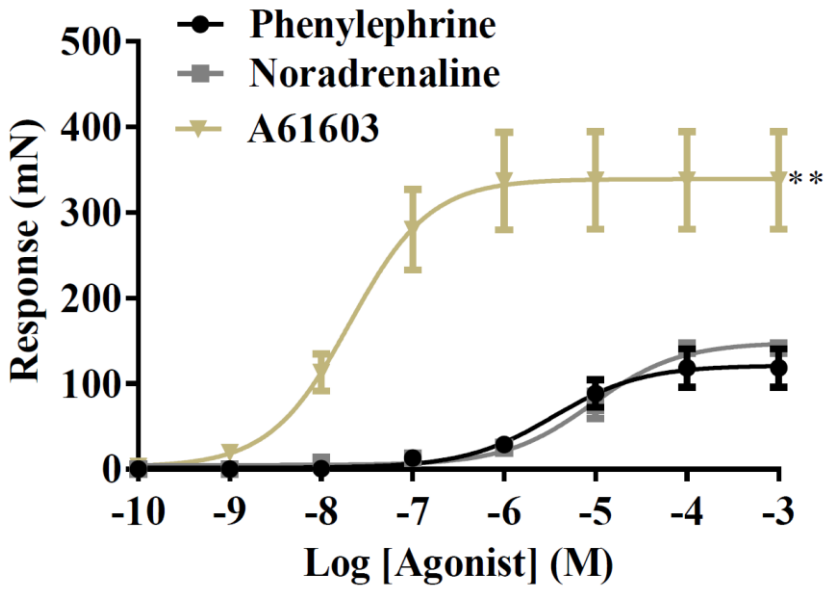


Figure 4.1 Concentration-response curves to phenylephrine, noradrenaline and A61603 in urethral tissues of the pig. **p<0.01 vs. response to noradrenaline (one way ANOVA, with Tukey post hoc test); n=4-10.

The effect of urothelium/lamina propria on receptor-mediated responses

Removal of the urothelium/LP resulted in increased maximum contractions to A61603, phenylephrine and noradrenaline (Table 4.2, Figure 4.2; $p<0.05$). The percentage inhibition of contractile responses by the urothelium/LP was similar for all agonists (Figure 4.2).

Comparison of the role of the urothelium/lamina propria in regions of the urethra

The urothelium/LP significantly depressed responses by a similar amount to phenylephrine in both the proximal ($p<0.05$) and mid ($p<0.001$) urethra (Table 4.3). However, the effect was not observed in the distal region of the urethra. The urothelium/LP had no significant effect on pEC_{50} values for phenylephrine in the mid and distal urethral tissue. However, in the presence of urothelium/LP, the pEC_{50} values for phenylephrine in the proximal urethra were increased, showing increased potency (Figure 4.3; $p<0.001$).

Effect of age on the role of the urothelium/lamina propria in the proximal urethra

Removal of the urothelium/LP from urethral tissues from young and older pigs resulted in significantly greater maximum contractile responses of urethral tissues to phenylephrine, (Table 4.4, Figure 4.4; n=6-7). The amount of inhibition caused by the presence of the urothelium/LP was similar in the tissues from the young and older pigs. Moreover, the pEC_{50} values for phenylephrine in tissues with urothelium/LP were akin to those in tissues without urothelium/LP in both young and older pigs.

Influence of urothelium/lamina propria on desensitisation of concentration-response curves to phenylephrine

The urothelium/LP had no significant effect on the desensitisation or repeatability of responses to phenylephrine. Responses to subsequent additions of phenylephrine were significantly desensitised both in the presence or absence of urothelium/LP, and desensitisation of responses was similar in the young and the older pig tissues (Table 4.5; Figure 4.5; n=6). Furthermore, the pEC_{50} values for phenylephrine were not affected by the removal of the urothelium/LP and by the subsequent addition of phenylephrine in the young or old tissues.

	Noradrenaline		Phenylephrine		A61603	
	Smooth muscle + urothelium/LP	**Smooth muscle only**	**Smooth muscle + urothelium/LP**	**Smooth muscle only**	**Smooth muscle + urothelium/LP**	**Smooth muscle only**
Max response (mN)	141.3±8.8	**429.5±80.0***	139.4±28.5	**238.1±23.6***	337.7±57.0	**913.6±225.6***
pEC_{50}	5.0±0.1	5.3±0.2	5.4±0.3	5.5±0.2	7.7±0.3	7.6±0.4
Percentage decrease in maximum (%)	67.1±20.0%		41.5±12.8%		63.0±24.8%	
n	4		7		10	

Table 4.2 Effect of the urothelium/LP on urethral responses. Mean (±SEM) maximum contractile responses and pEC_{50} values for noradrenaline, phenylephrine and A61603 on urethral tissues (proximal, 36 months) (+/- urothelium/LP). *p<0.05 vs. smooth muscle with urothelium/LP (Student's t-test). LP=lamina propria.

	Proximal		Mid		Distal	
	Smooth muscle only	**Smooth muscle + urothelium/LP**	**Smooth muscle only**	**Smooth muscle + urothelium/LP**	**Smooth muscle only**	**Smooth muscle + urothelium/LP**
Max response (mN)	151.2±8.6	**56.2±10.5***	125.1±5.4	**53.2±2.6*****	79.9±7.7	64.3±5.8
pEC_{50}	5.3±0.1	**6.3±0.2*****	5.5±0.1	5.7±0.1	5.3±0.1	5.5±0.1
Percentage decrease in maximum (%)	63.2±19.1%		58.4±12.6%		19.5±8.4%	
n	8		13		9	

Table 4.3 Effect on region of urethral responses. Mean (±SEM) maximum contractile responses and pEC_{50} values for phenylephrine for various regions of the urethra (36 months) (+/- urothelium/LP). *p<0.05, ***p<0.001 vs. smooth muscle only response (Student's t-test). LP=lamina propria.

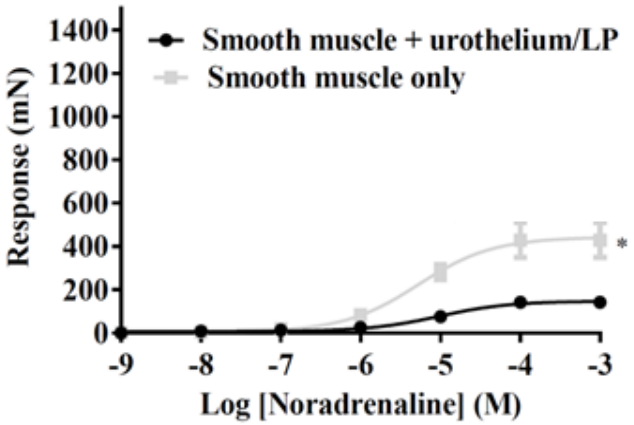
	Young		Old	
	Smooth muscle + urothelium/LP	**Smooth muscle only**	**Smooth muscle + urothelium/LP**	**Smooth muscle only**
Max response (mN)	90.6±2.2	**140.3±6.0*** **	104.2±13.7	**191.6±18.5***
pEC_{50}	5.4±0.1	5.6±0.1	5.5±0.3	5.5±0.3
Percentage decrease in maximum (%)	34.3±3.6		46.7±17.3	
n	6		7	

Table 4.4 Effect of age on the role of the urothelium/LP. Mean (±SEM) maximum contractile responses and pEC_{50} values for phenylephrine for the young and older urethral tissues (proximal) (+/- urothelium/LP). *p<0.05, ***p<0.001 vs. smooth muscle with urothelium/LP response (Student's t-test). LP=lamina propria.

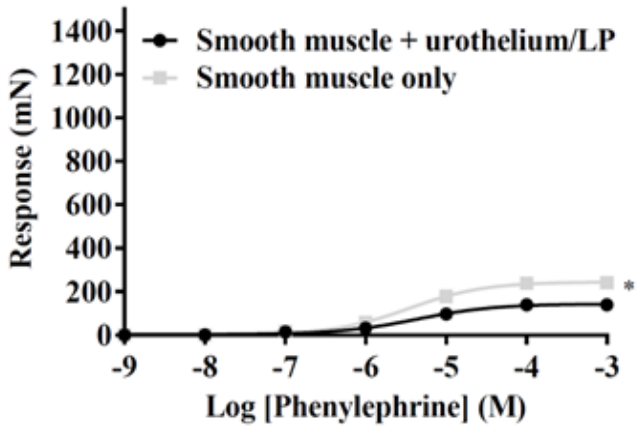
	Young				Old			
	Smooth muscle only		**Smooth muscle + urothelium/ LP**		**Smooth muscle only**		**Smooth muscle + urothelium/ LP**	
	1st Curve	**2nd Curve**	**1st Curve**	**2nd Curve**	**1st Curve**	**2nd Curve**	**1st Curve**	**2nd Curve**
Max response (mN)	140.3±6.0	**62.9±10.5 ****	90.6±2.2	**54.0±10.9***	167.5±12.1	**90.4±15.9 *****	133.1±19.3	**67.7±14.9 *****
pEC_{50}	5.6±0.1	5.8±0.6	5.4±0.1	5.5±0.5	5.5±0.2	5.5±0.4	5.6±0.4	5.7±0.6
Percentage decrease in maximum (%)	56.1±9.5		42.9±16.6		50.9±8.1		49.9±6.9	
n	6		6		6		6	

Table 4.5 Influence of urothelium/LP on desensitisation of urethral responses. Mean (±SEM) maximum contractile responses and pEC_{50} values for phenylephrine in proximal tissues from young and older pigs (+/- urothelium/LP). *p<0.05, **p<0.01, ***p<0.001 vs 1st response curve (Paired Student's t-test). LP=lamina propria.

Noradrenaline



Phenylephrine



A61603

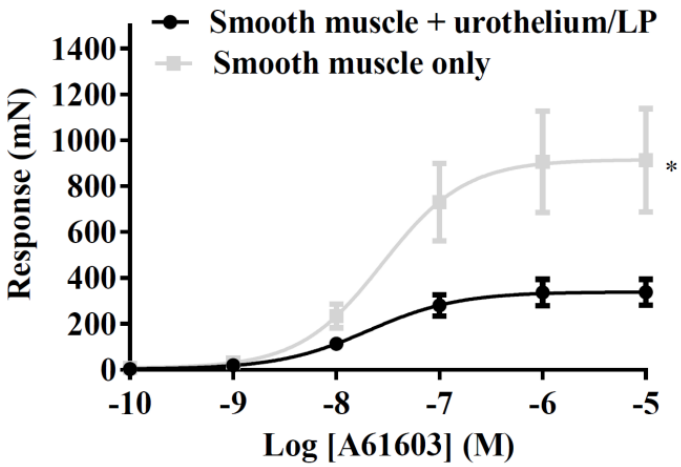
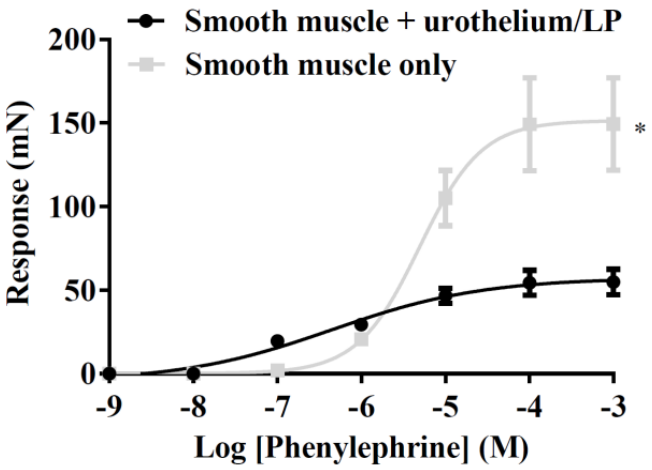
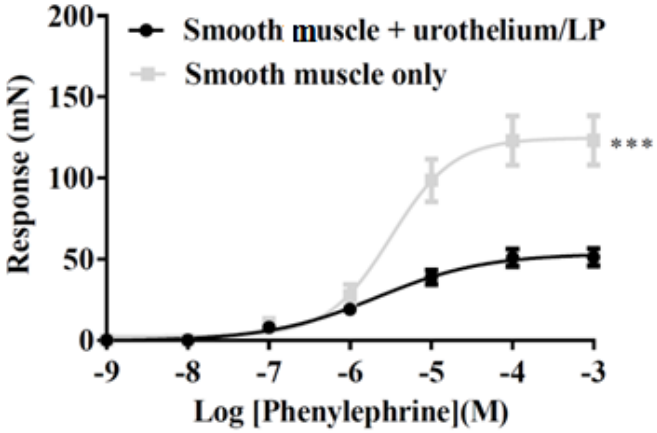


Figure 4.2 Concentration-response curves of proximal urethral tissues (36 months, +/- urothelium/LP) to noradrenaline, phenylephrine and A61603. *p<0.05 vs. smooth muscle with urothelium/LP response (Student's t-test); n=4-10. LP=lamina propria.

Proximal



Mid



Distal

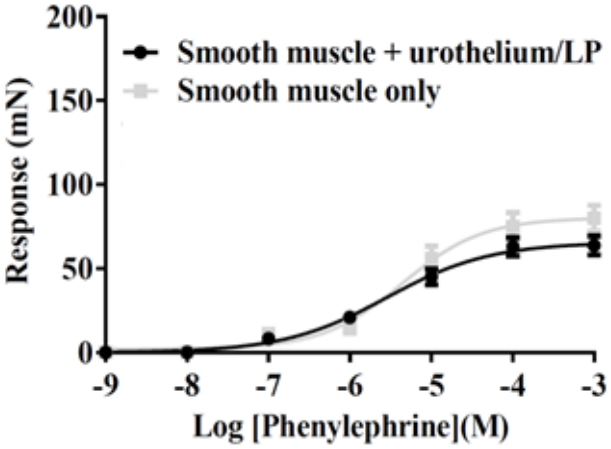


Figure 4.3 Concentration-response curves to phenylephrine for urethral tissues (36 months, +/- urothelium/LP) from various regions of the urethra. n=8-13. *p<0.05, ***p<0.001 vs. smooth muscle with urothelium/LP response (Student's t-test). LP=lamina propria.

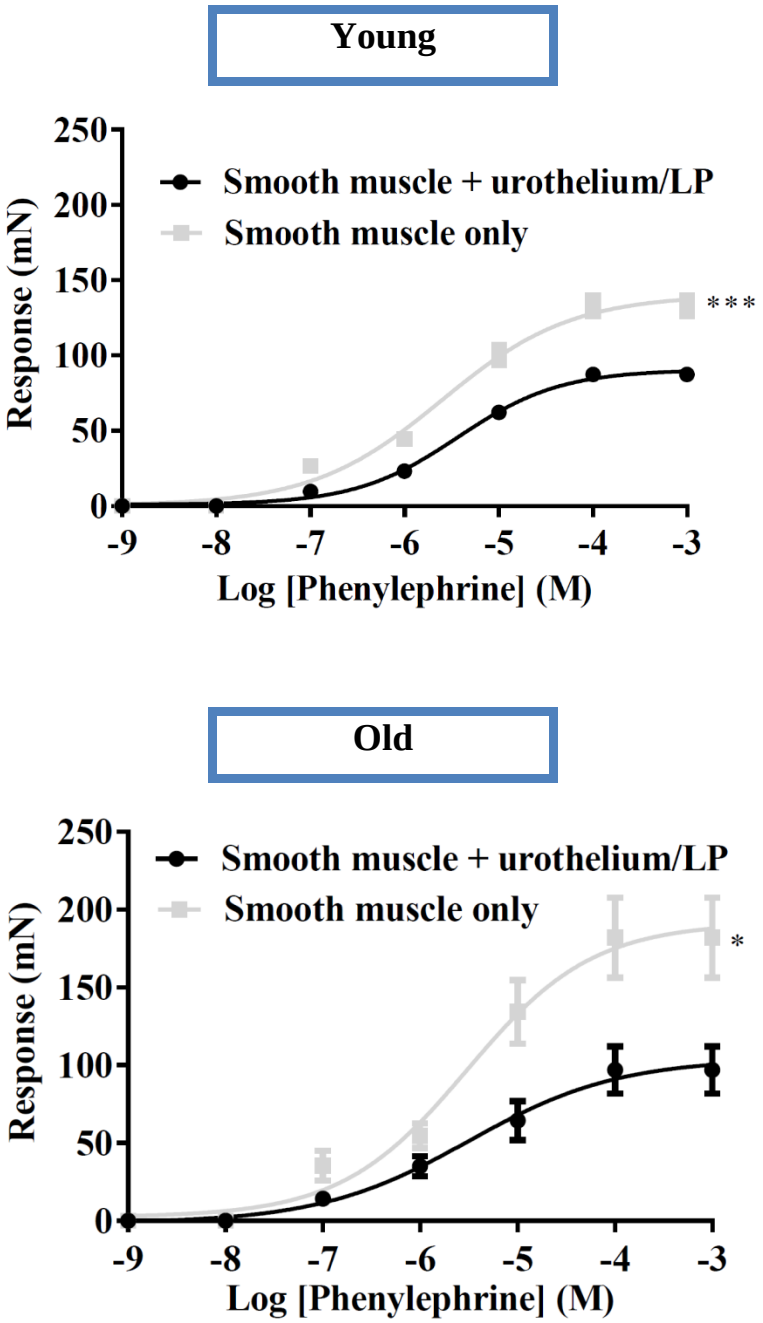


Figure 4.4 Concentration-response curves of proximal urethral tissues (+/- urothelium/LP) from young and older pigs to phenylephrine. n=6-7. *p<0.05, ***p<0.001 vs. smooth muscle with urothelium/LP response (Student's t-test). LP=lamina propria.

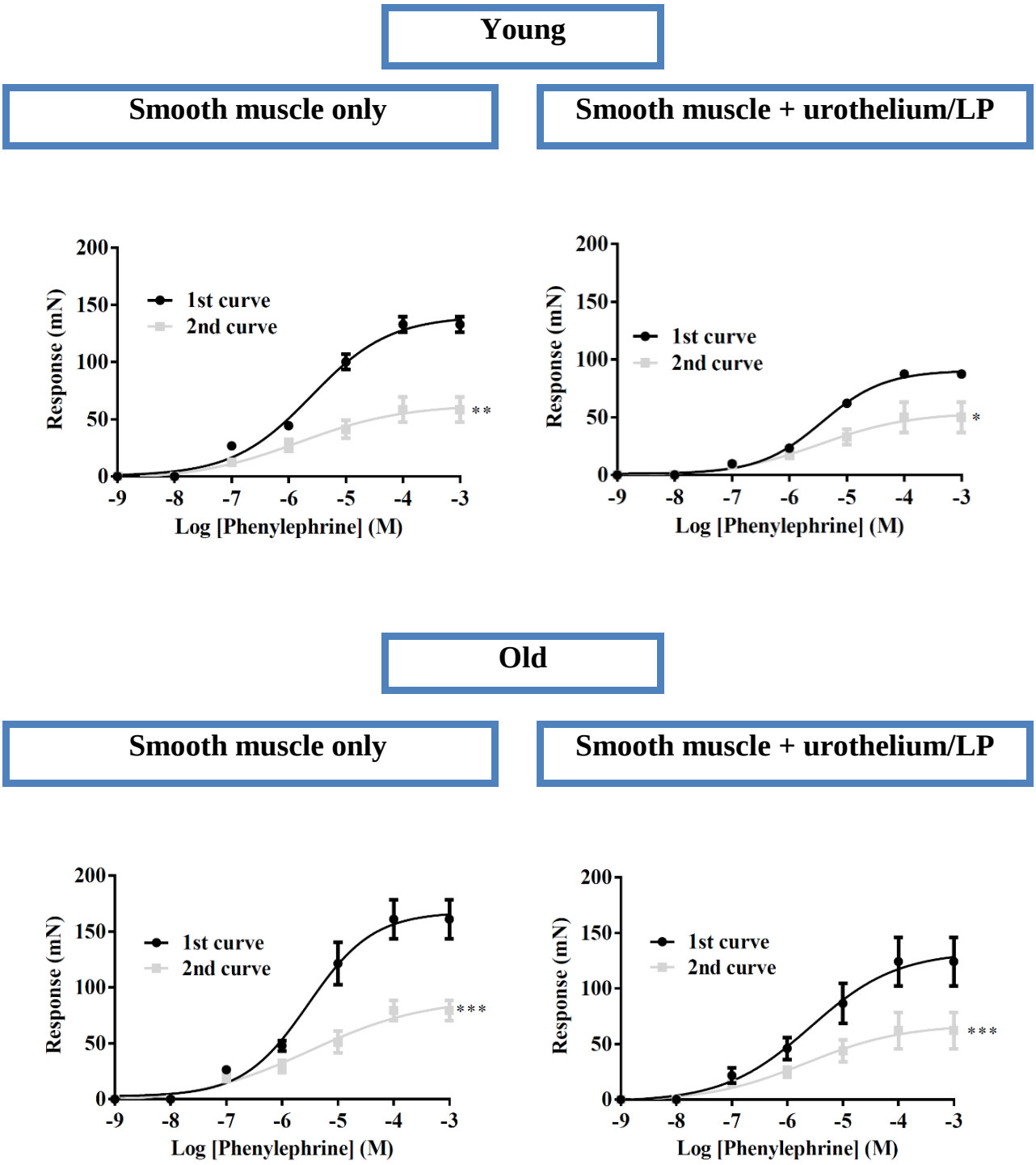


Figure 4.5 Mean concentration-response curves for urethral tissues (proximal) from young and older urethral tissues (+/- urothelium/LP) to phenylephrine; n=6.*p<0.05, **p<0.01, ***p<0.001 vs 1st response curve (Paired Student's t-test). LP=lamina propria.

Influence of urothelium/lamina propria on muscarinic receptor-mediated responses of the porcine urethra

Maximum contractile responses of urethral tissues without urothelium/LP to cumulative concentrations of carbachol were significantly greater than those with urothelium/LP (Table 4.6; Figure 4.6; n=5, $p<0.01$). The pEC_{50} values for carbachol for tissues with urothelium/LP were similar to those of tissues without urothelium/LP.

	Carbachol	
	Smooth muscle + urothelium/LP	**Smooth muscle only**
Max response (mN)	49.8±7.6	**141.0±25.1****
pEC_{50}	5.8±0.2	5.2±0.2
Percentage decrease in max (%)	64.7±22.6	
n	5	

Table 4.6 Mean (±SEM) maximum contractile responses and pEC_{50} values for carbachol for proximal urethral tissues from 36 months old pig (+/- urothelium/LP). ** P<0.01 vs. smooth muscle with urothelium/LP (Student's t-test). LP=lamina propria.

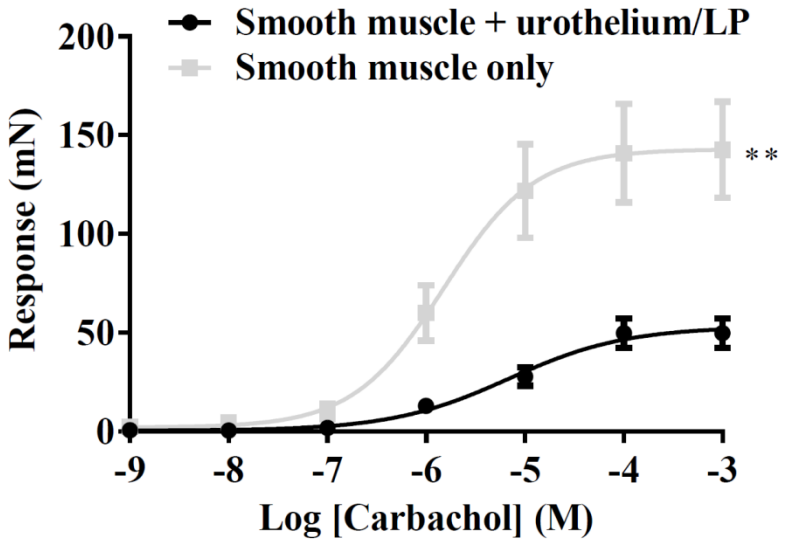


Figure 4.6 Concentration-response curves of proximal urethral tissues (36 months, +/- urothelium/LP) to carbachol; n=5. ** P<0.01 vs. smooth muscle with urothelium/LP (Student's t-test). LP=lamina propria.

Influence of the urothelium/lamina propria on ß-AR-mediated responses of the porcine urethra

Cumulative concentrations of isoprenaline caused concentration-dependent relaxations of the porcine urethral from baseline (Table 4.7, Figure 4.7). Tissues without urothelium/LP-relaxed to a significantly greater extent to isoprenaline than tissues with an intact urothelium/LP ($p<0.05$). Moreover, the pEC_{50} values for isoprenaline were similar for tissues with and without urothelium/LP.

	Isoprenaline	
	Smooth muscle + urothelium/ LP	**Smooth muscle only**
Max relaxation response (mN)	89.3±6.1	**131.3±13.8***
pEC_{50}	7.2±0.2	7.0±0.2
Percentage inhibition of relaxation (%)	32.0±12.8%	
n	10	

Table 4.7 Mean (±SEM) maximum relaxation responses and pEC_{50} values for isoprenaline in proximal urethral tissues (36 months, +/- urothelium/LP). *$p<0.05$ vs. smooth muscle with urothelium/LP (Student's t-test); n=10. LP=lamina propria.

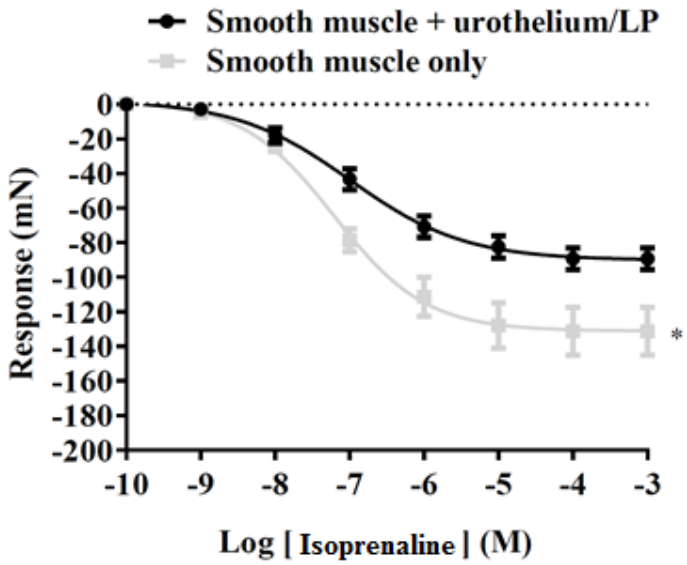


Figure 4.7 Concentration-response curves to isoprenaline for proximal urethral tissues (36 months, +/- urothelium/LP). *$p<0.05$ vs. smooth muscle with urothelium/LP (Student's t-test); n=10. LP=lamina propria.

Role of prostaglandins and nitric oxide in urothelium/lamina propria inhibition in the urethra

Incubation with LNNA (100μM) had no effect on proximal urethral responses to noradrenaline (Table 4.8; Figure 4.8), phenylephrine (Table 4.8; Figure 4.9) and A61603 (Table 4.8; Figure 4.10) irrespective of whether with urothelium/LP was present or not. The presence of indomethacin (10μM) also had no effect on responses, except for urethral tissues without urothelium/LP, where contractions to phenylephrine (Table 4.8; Figure 4.9; $p<0.05$), but not other agonists, were reduced by indomethacin.

	Smooth muscle only		Smooth muscle + urothelium/LP		Smooth muscle only		Smooth muscle + urothelium/LP	
Noradrenaline	**Control**	**+ Indomethacin (10µM)**	**Control**	**+ Indomethacin (10µM)**	**Control**	**+ LNNA (100µM)**	**Control**	**+ LNNA (100µM)**
Max response (mN)	525.5±49.0	542.8±20.4	228.2±12.4	180.6±19.9	523.8±36	486.6±70.0	167.3±17.7	186.8±14.5
pEC_{50}	5.8±0.3	5.9±0.1	5.8±0.2	5.7±0.3	6.0±0.2	6.0±0.4	6.0±0.3	5.9±0.2
Percentage inhibition			52.7±6.7	66.1±7.5			67.3±5.3	56.2±8.5
n	5		5		5		5	
Phenylephrine								
Max response (mN)	426.4±38.8	**196.6±23.2***	183.1±16.4	231.0±27.9	263.1±26.0	346.7±26.3	217.4±21.3	160.0±13.9
pEC_{50}	5.9±0.3	5.8±0.3	5.5±0.2	5.5±0.3	5.6±0.3	5.7±0.2	5.7±0.3	5.7±0.2
Percentage inhibition			55.5±8.8	58.4±20.3			45.9±21.2	53.2±3.2
n	5		5		5		5	
A61603								
Max response (mN)	772.0±85.6	1022±102.2	297.0±67.7	321.8±65.1	804.9±117.0	872.2±194.7	334.6±57.6	197.1±51.6
pEC_{50}	7.1±0.4	7.4±0.3	7.5±0.9	7.4±0.7	7.0±0.5	7.4±0.8	7.2±0.6	6.7±0.7
Percentage inhibition			63.0±5.7	66.3±8.2			57.8±6.8	71.5±6.5
n	5		5		5		5	

Table 4.8 Effect of LNNA and indomethacin on urethral responses. Mean (±SEM,) pEC_{50} values for agonists and maximum contractions of porcine urethral tissues (+/- urothelium/LP) to noradrenaline, phenylephrine and A616063 in the presence or absence of cyclooxygenase inhibitor (indomethacin (10µM)) or nitric oxide synthase inhibitor (LNNA (100µM)). LP=lamina propria.

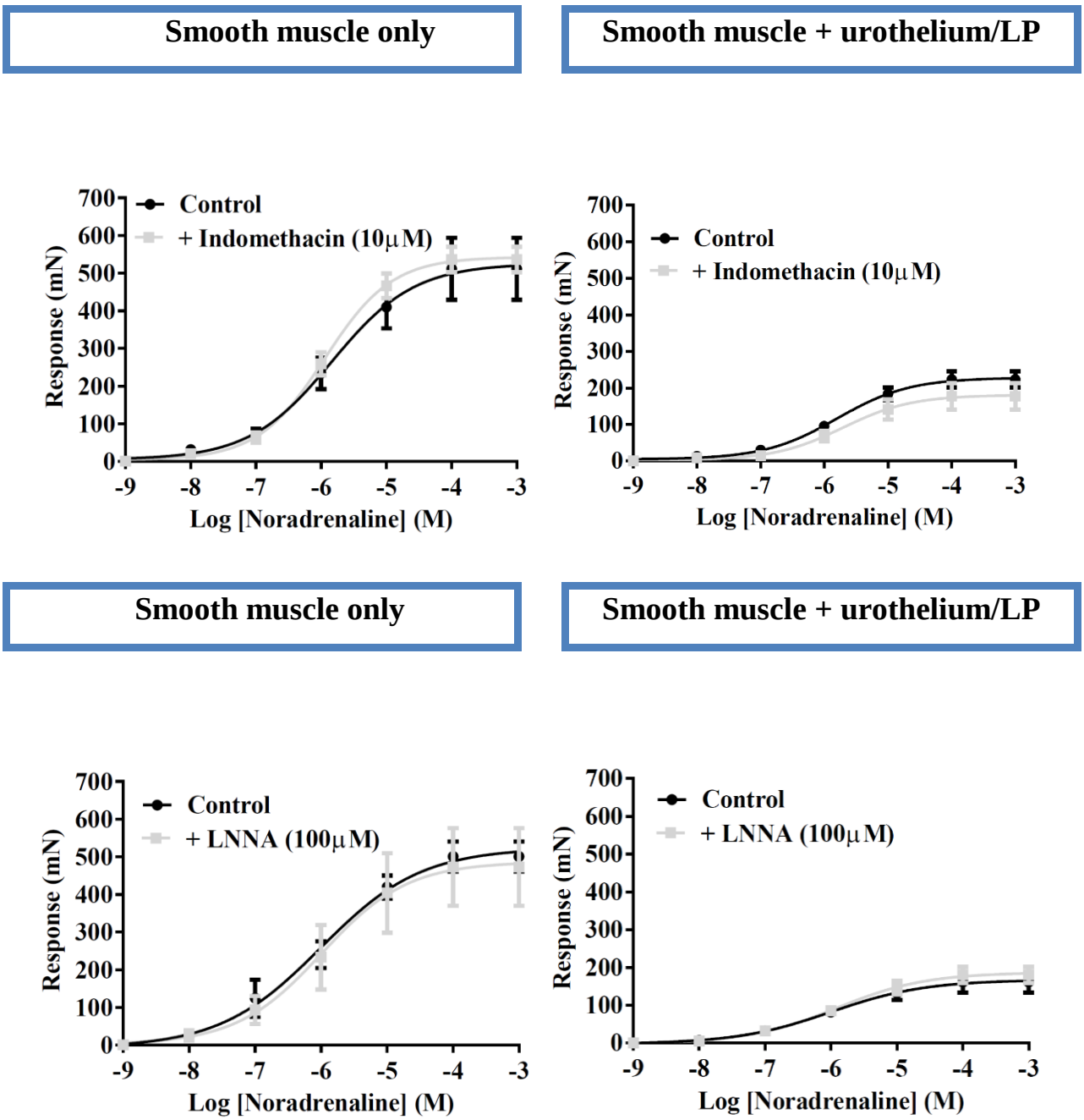


Figure 4.8 Concentration-response curves of porcine urethral tissues (+/-urothelium/LP) to noradrenaline in the presence or absence of cyclooxygenase inhibitor (indomethacin (10µM)) or nitric oxide synthase inhibitor (LNNA (100µM)); n=5. LP=lamina propria.

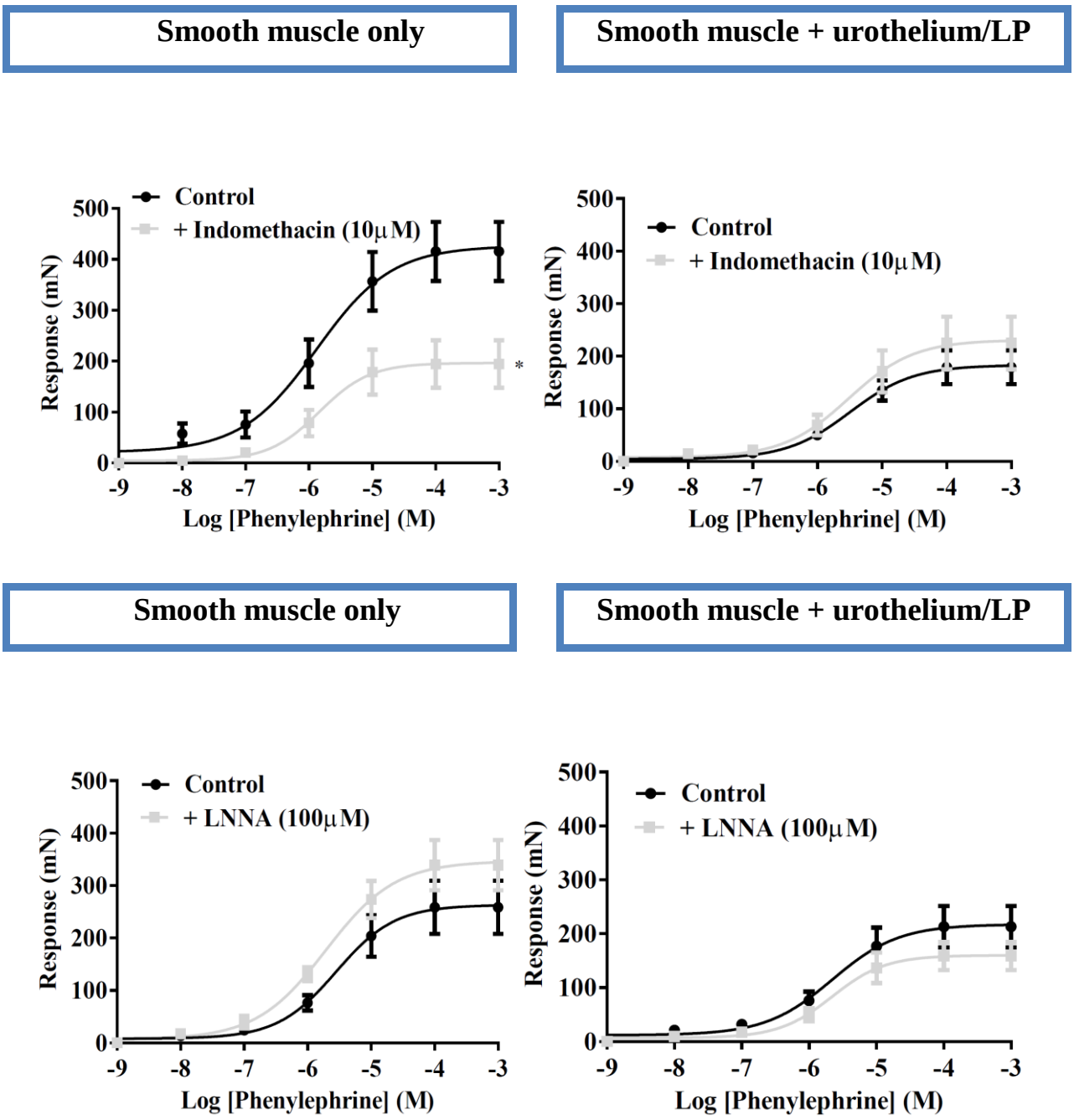


Figure 4.9 Concentration-response curves of porcine urethral tissues (+/-urothelium/LP) to phenylephrine in the presence or absence of indomethacin (10μM) or LNNA (100μM). *p<0.05; n=5. LP=lamina propria.

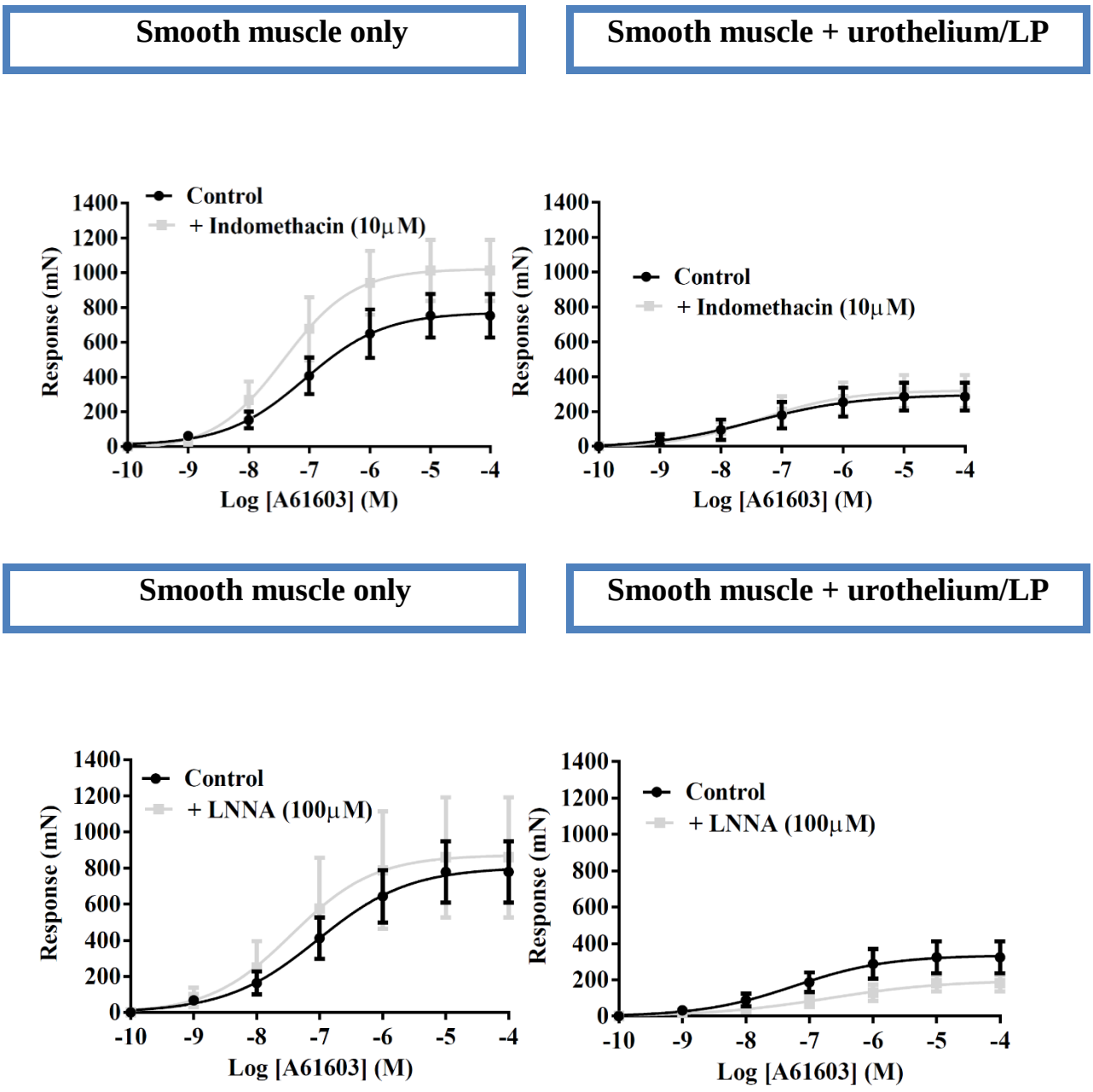


Figure 4.10 Concentration-response curves of porcine urethral tissues (+/-urothelium/LP) to A61603 in the presence or absence of cyclooxygenase inhibitor (indomethacin (10μM)) or nitric oxide synthase inhibitor (LNNA (100μM)); n=5. LP=lamina propria.

4.4 DISCUSSION

The experiments conducted in this chapter were performed to investigate the influence of the urothelium/LP on AR and mAChR-mediated responses of porcine urethral tissues. Contractions of the normal porcine urethra to carbachol are mediated by M_2 and M_3 mAChRs in circular muscle but by M_3 mAChRs in the longitudinal muscle (Yamanishi et al., 2002d). Similarly, urethral tissue from human and animals expresses β-ARs (Morita et al., 2000; Yamanishi et al., 2002c; 2003a), which mediate relaxation (Takeda et al., 2003; Yamanishi et al., 2003a). However, the effects of exogenous β-AR agonists on urethral tissues are small relative to those of the bladder (Takeda et al., 2003). So far, the physiological role of the mAChRs and β-ARs in the urethra is still a matter for debate given the major role of the α_1-AR.

In this study, activation of β-ARs with isoprenaline relaxed the urethral tissues, while carbachol contracted the urethral tissues. This supports previous work (Alexandre et al., 2016 and Giglio & Tobin, 2009; Kedia et al., 2013). Moreover, mAChR-mediated responses were significantly less than α_1-AR-mediated responses. Ek and colleagues, (1977) reported that acetylcholine produced only a weak contraction in the isolated human urethra, and this effect was blocked by atropine, suggesting that mAChRs mediated the responses. Kedia et al., (2013) showed that acetylcholine and carbachol had only minor contractile effects on the human male urethral smooth muscle. Therefore, this finding is in accordance with earlier reports and suggests only a minor role for mAChR in contraction of the urethra.

Does the urothelium/lamina propria affect the receptor-mediated responses of the urethra?

The inhibitory effects of the urothelium/LP on smooth muscle contractions have been shown to involve a receptor-mediated mechanism in the bladder (Templeman et al., 2002; Chaiyaprasithi et al., 2003). Therefore, it was hypothesised that stimulation of α-ARs and mAChRs would trigger a similar inhibitory effect via the urothelium/LP, and depress urethral smooth muscle responses. It was observed that the urethral urothelium/LP indeed did have an inhibitory effect on receptor-mediated responses. The receptor-mediated responses were significantly greater in tissues without urothelium/LP compared to those with their

urothelium/LP intact for all agonist (AR and mAChR agonist). The inhibitory effect seems to be similar in size to that reported in the porcine bladder in response to carbachol (Hawthorn et al. 2000). Previous reports have shown that the urothelium/LP depressed receptor-mediated response in the rat (Fovaeus et al., 1999), guinea-pig (Guan et al., 2014a), cat (Levin et al., 1995), pig (Hawthorn et al. 2000; Templeman et al., 2002) and human (Chaiyaprasithi et al., 2003) detrusor smooth muscle only. The smaller responses in urethral strips with urothelium/LP could be argued to be due to poor agonist penetration through urothelium/LP. However, the muscle is exposed to the agonist on all sides of the strips. However, Templeman et al., (2002) showed that in the presence of an intact urothelium, contractile responses to phenylephrine were not depressed in the pig bladder trigone. The inability of phenylephrine to induce the inhibitory effect of the urothelium/LP may be associated with the lower contribution of α_1-ARs to bladder contraction (Nomiya & Yamaguchi, 2003). In the present study, the inhibitory effect in urethra was observed with stimulation by mAChR and α_1-AR agonists. Thus this report shows for the first time that the urothelium/LP has an inhibitory effect on receptor-mediated responses in the porcine urethra.

In addition, previous reports showing inhibitory effects of the urothelium/LP have not compared inhibitory effect of the urothelium/LP in relation to α_1-AR agonist efficacy, affinity and potency, thereby making this report the first to do so. The effects of the urothelium/LP on receptor-mediated responses were similar for the α_1-ARs agonist irrespective of their affinity, efficacy and potency. Moreover, the effects of urothelium/LP on receptor-mediated responses were similar for the α_1-AR- and mAChR-mediated responses, thus suggesting that the inhibitory effect may not be receptor-type dependent in the porcine urethra.

Effect of the urothelium/lamina propria on β-adrenoceptor-mediated relaxation responses

In the present study, isoprenaline-induced relaxations were significantly depressed in tissues with urothelium/LP. Since there are no reports showing the effect of urothelium/LP on β-AR-mediated responses in the urethra, the comparison could only be made with responses in the bladder. Murakami et al., (2007) showed that maximum relaxation to isoprenaline and potency was similar in the absence and presence of the urothelium/LP in the pig bladder. Although this report was based on the relaxation of carbachol pre-contracted bladder strips,

whereas relaxation of basal tension was shown in the present study, this suggests a difference in the role of urothelium/LP between the bladder and urethra in response to β-ARs activation. The attenuated relaxation to isoprenaline in the presence of the urothelium/LP could indicate the release of a contractile agent from the urothelium/LP. Such an agent may include ATP (Young et al., 2012), which is known to be released from urothelium, and which acts on P2Y or P2X receptor, or acetylcholine (Hanna-Mitchell et al., 2007; Yoshida et al., 2006) which acts on the mAChR. Moreover, activation of the P2Y receptor may mediate further ATP release from the urothelium (Hanna-Mitchell et al., 2007).

The possibility of the urothelium releasing a 'contracting factor' in addition to the 'inhibitory factor' has been suggested previously albeit in the bladder (Propping et al., 2013; 2015a, b). Such a phenomenon may exist in the urethra, and a possibility is a contractile prostaglandin (Downie & Karmazyn, 1984) which may act on receptors on the smooth muscle or urothelium/LP to mediate the depression of isoprenaline-mediated relaxation. Prostaglandin E_2 has been shown to contract animal and human bladder smooth muscle strips (Root et al., 2015) and the expression of the prostaglandin E receptors, EP_1, EP_2, EP_3, and EP_4, and mRNA for EP_2, EP_3, and EP_4 have been reported in dog urethral urothelium (Ponglowhapan et al., 2010). Reductions in bladder capacity have been attributed to one or more of the contractile prostanoid receptor subtypes EP_1, EP_2 and EP_3 in cells, animal models and human (Schroder et al., 2004; Wang et al., 2008; Su et al., 2008b; Palea et al., 1998). Furthermore, inhibition of the EP_1 prostanoid receptor has been shown to increase the capacity of rat bladder in vivo (Lee et al., 2007). Thus, activation of prostaglandin E receptors via a contractile prostaglandin released by the urothelium may also be responsible for depression of isoprenaline-mediated relaxation reported in this study.

Influence of urothelium/lamina propria in different urethral regions and effect of age

Templeman et al., (2002) reported the inhibitory effect of the urothelium/LP on receptor-mediated responses in the pig trigone but did not investigate the urethra or the regions. We found that the urothelium/LP of the proximal and the mid-urethra had a greater inhibitory effect on phenylephrine-induced contractile responses compared to that in the distal urethra. This difference in the inhibitory effect between the proximal, mid and distal urethral tissues

may reflect differences in innervation (Yoshimura et al., 2003). The proximal urethra/bladder neck is densely innervated (Sienkiewicz et al., 2004) and the bladder neck/proximal urethra helps to maintain continence. Therefore, this result suggests the proximal urethra acts as a possible centralised region for neural control and modulation of the luminal pressure of the urethra as suggested by Pidsudko, (2004).

Investigation of factors mediating the urothelium/lamina propria inhibitory effects

Previous attempts to elucidate the nature of urothelium derived inhibitory factor in the bladder have been unsuccessful (Chaiyaprasithi et al., 2003; Guan et al., 2014a; Hawthorn et al., 2000). Agents that may inhibit smooth muscle contraction and which are released from the urothelium include prostanoids (Guan et al., 2014b) and NO (Andersson et al., 2012; Birder et al., 1998; 2001; Kullmann et al., 2008a). NO is released from urothelium in response to β-AR activation via NOS (Birder et al., 2002b). NO is released from the urothelium upon activation of mAChRs, and this NO can directly or indirectly attenuate detrusor smooth muscle contractility (Andersson et al., 2012; Kullmann et al., 2008a). Prostanoids are also produced locally within the bladder urothelium in human and other species (Kang et al., 2013; 2015a; 2015b; McDermott et al., 2012; 2013; Nile & Gillespie, 2012; Wheeler et al., 2002) and are produced in response to physical stretch (Downie & Karmazyn, 1984; Kang et al., 2013; 2015a; 2015b; McDermott et al., 2013). Nile & Gillespie, (2012) showed that activation of M_2 -mAChR induced production of prostaglandins. Whilst urothelium-derived inhibitory factor in the bladder has been shown not to be nitric oxide or prostaglandins, the possibility of either nitric oxide or prostaglandins or both mediating the urothelium/LP inhibitory effect was a possibility in the urethra.

Inhibition of NOS and cyclooxygenase with LNNA and indomethacin respectively did not affect the inhibitory effect of the urothelium/LP. Thus our result is similar to that reported in the bladder of guinea-pig (Guan et al., 2014a), pig (Hawthorn et al., 2000) and human (Chaiyaprasithi et al., 2003).

Other possible agents that could mediate the inhibitory effect include ATP, hydrogen sulphide (H_2S) and carbon monoxide. Gai et al., (2013) reported the expression of all the

three synthases of endogenous H_2S, (cystathionine-β-synthethase, cystathionine-γ-lyase and 3-mercaptosulfutransferase) in the urothelium of human and rat urethra. ATP could also mediate the urothelium inhibitory effect via the A2A, P_1 receptors after breakdown to adenosine or via $P2Y_1$ receptors after breakdown to ADP (Hernandez et al., 2009). Moreover, relaxation may be associated with K^+ channels opening and activation of guanylate cyclase since these relaxing transmitters (carbon monoxide, ATP and H_2S), mediate relaxation by opening of the K^+ channels (Li et al., 2008; Naik & Walker et al., 2003) and activation of guanylate cyclase (Zakhary et al., 1997). Although it is not possible to comment on the likelihood of the candidates mediating the inhibitory effect in the urethra in the present study, in the bladder, the inhibitory agents appears to be neither NO, a cyclooxygenase product, a catecholamine, adenosine, cyclic GMP, γ-aminobutyric acid nor an endothelium-derived hyperpolarizing factor sensitive to apamin (inhibitor of the small-conductance Ca^{2+}-activated K^+-channels) because urothelium-induced inhibition of contractions was not prevented in the presence of L-NOARG, methylene blue, indomethacin, propranolol, ODQ, suramin, TEA or apamin (Chaiyaprasithi et al., 2003; Hawthorn et al., 2000). These drugs inhibit NOS, guanylate cyclase, cyclooxygenase, β-adrenoceptors, guanylate cyclase, P_2 receptors, K^+ channel and small conductant Ca^{2+}-activated K^+ channel respectively. Thus whether further study in the urethra elucidates the candidate is not known.

An additional finding in the present study was that cyclooxygenase inhibition with indomethacin significantly reduced urethral smooth muscle contractility to phenylephrine. This result suggests that phenylephrine induces α_1-AR-mediated prostaglandin release from the urethral smooth muscle and that the prostaglandin release had a contractile effect on the urethral smooth muscle.

Prostaglandin was not released from the urothelium, but the urethral smooth muscle because indomethacin had no effect on responses of tissues with urothelium. Earlier we have shown that the urothelium had an inhibitory effect on the contractile responses of the urethral smooth muscle in response to α_1-ARs activation with noradrenaline, phenylephrine and A61603. Moreover, the urothelium has an inhibitory effect on smooth muscle in the bladder and the inhibitory effect is mediated by the release of factors which are diffusible within a short and longer distance (Chaiyaprasithi et al., 2003; Guan et al., 2014a; Hawthorn et al. 2000; Templeman et al., 2002). The absence of the effect of the indomethacin in urethral

tissues with urothelium suggests that the urothelium inhibitory effect masked the prostaglandin contractile effect. Moreover, the effect of indomethacin was seen only in phenylephrine-contracted tissues which suggested that the release of a contractile prostaglandin may depend on activation of receptor-coupled pathways, which differ between agonists (discussed in chapter 5).

Prostaglandins can be released upon α_1-AR activation, as shown in the rabbit (Yousufzai & Abdel-Latif, 1983; 1984) and bovine iris (Yousufzai & Abdel-Latif, 1983). Analysis of tissue prostaglandin E_2 revealed that rabbit and bovine iris prostaglandin E_2 content was increased following 15 minutes incubation with an α_1-AR agonist (Yousufzai & Abdel-Latif, 1983). The release of prostaglandin E_2 was time-dependent and the agonist-induced release of prostaglandin E_2 was abolished by low concentrations of indomethacin, thus confirming the release of prostaglandin E_2 via activation of cyclooxygenase.

Phenylephrine stimulation of prostaglandin release in the porcine urethra is probably mediated through α_1-ARs and maximal adrenoceptor stimulation may require the presence of Ca^{2+} as shown for rabbit and bovine iris (Yousufzai & Abdel-Latif, 1983). Moreover, phenylephrine may induce the responses via the increase of the breakdown of phosphatidylinositol which may be accompanied by a significant increase in the release of arachidonic acid and consequently prostaglandin as shown in the rabbit iris (Yousufzai & Abdel-Latif, 1984).

α_1-AR agonists are prescribed off-label for the therapeutic increase of urethral tone, and agonist-induced increase in prostaglandin synthesis may play a significant role in mediating the pharmacological effects of these therapeutic agents.

Does the urothelium/lamina propria have an effect on desensitisation of α_1-adrenoceptor-mediated responses?

In the previous chapter, it was shown that responses to agonists that have a high affinity for α_1-ARs (phenylephrine and A61603) are desensitised to a greater extent than responses to noradrenaline. In this chapter, we investigated whether the presence of the urothelium affects desensitisation of α_1-AR responses.

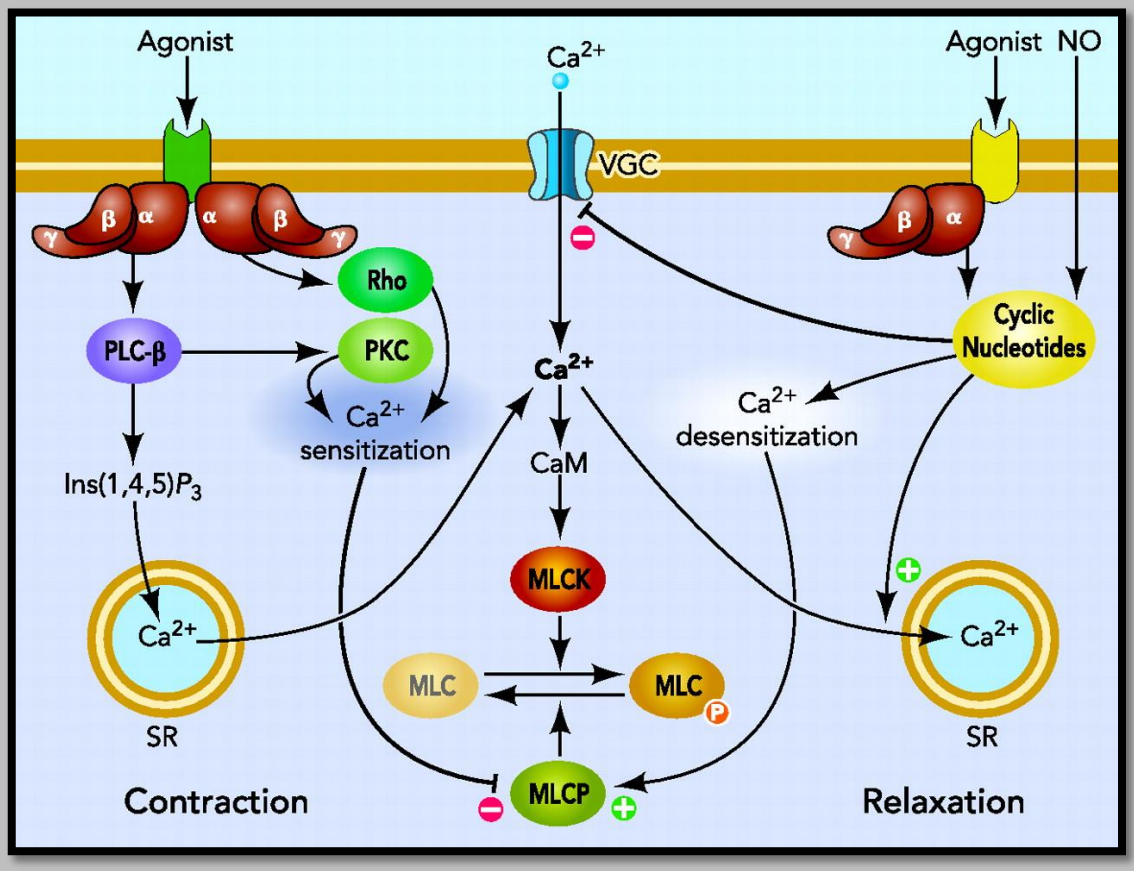
The urothelium/LP had no significant effect on desensitisation of the subsequent contractile responses of the urethral tissues to phenylephrine. Moreover, these observations were similar between the young and older urethral tissues. Ferguson et al., (2015) showed that the urothelium could increase desensitisation of receptors, albeit for purinergic receptors. They showed urothelial $P2X_3$ mediated desensitisation of detrusor responses in the rat, and explained this observation by a $P2X_3$ receptor driven paracrine amplification of ATP release from umbrella cells, to increase afferent transmission in the suburothelial sensory plexus and desensitisation of $P2X_1$-mediated purinergic detrusor contractions (Ferguson et al., 2015). The finding that the urothelium/LP does not influence α_1-AR desensitisation, suggests that desensitisation of α_1-AR is responsible for failure of α_1-AR agonist in the treatment of stress urinary incontinence clinically, the mechanism does not appear to involve the urothelium and must lie at the level of receptor-mediated signalling pathways.

Presently, the importance of these results for normal urethral function is not entirely known. However, the urothelium/LP may play a significant role in the normal physiology of the urethra and the aetiology and pathophysiology of stress urinary incontinence.

Conclusion

- The urothelium/LP has an inhibitory effect on receptor-mediated responses of the porcine urethra.
- The inhibitory effect of the urothelium/LP is seen in all urethral regions except the distal portion.
- The influence of the urothelium/LP on phenylephrine-mediated responses is not affected by age.
- The inhibitory effect of the urothelium/LP on the urethral smooth muscle is not mediated by prostaglandins or NO.

CHAPTER 5



Modified from Puetz et al., 2009.

5 THE INTRACELLULAR PATHWAYS CONTRIBUTING TO URETHRAL CONTRACTILITY

5.1 BASAL SMOOTH MUSCLE TONE IN THE LOWER URINARY TRACT

Urethral closure pressure develops principally by contraction of the smooth and striated muscle (Shafik, 1999). However, an isolated rat urethral preparation was "continent" in the absence of external neural input, and flow ensued when the smooth muscle was relaxed (McHale et al., 2006). Furthermore, stimulation of the skeletal muscle made little difference to the ability of the contracted urethra to retain fluid and paralysing the striated muscle encircling the urethra of anaesthetized rats with D-tubocurarine did not result in urine leakage (Conte et al., 1991). Thus, it appears, that skeletal muscle is more important for resisting rapid pressure rises caused by coughing or laughing, than for maintaining a constant urethral tone, while the smooth muscle contributes greatly to the maintenance of basal tone (Bridgewater et al., 1993), maintaining urethral luminal pressure.

It is the circular smooth muscle of the urethra which exhibits intrinsic basal tone (Bridgewater et al., 1993), and this basal tone is resistant to neural blockade and mAChR antagonists, but sensitive to blockers of voltage-gated Ca^{2+} channels, and hence is myogenic (Brading et al., 1999; Bridgewater et al., 1993; Malmqvist et al., 2004; McKeag et al., 2001). The urethral basal tone can be enhanced by adrenergic and cholinergic input, or inhibited by non-adrenergic, non-cholinergic innervation, partially mediated by the transmitter NO (Bridgewater et al., 1993). Also, exogenously applied prostaglandin evoked a sustained increase in the rabbit urethral basal tone, suggesting a role of locally generated cyclooxygenase products (Ito & Kimoto, 1985). Moreover, Rho guanosine triphosphatases are involved in the generation of basal urethral tone (Malmqvist et al., 2004). To our knowledge, the pathways contributing to the urethra basal tone in the pig are not known and thus were the aim of the present study.

5.2 CALCIUM MOBILISATION IN THE LOWER URINARY TRACT

In the bladder, extracellular Ca^{2+} influx is mainly mediated by the opening of voltage-dependent L-type Ca^{2+} channels, which mediate detrusor contraction (Jiang et al., 2014) and spontaneous activity (Hashitani et al., 2001; 2004a, b). L-type Ca^{2+} channels have the greatest influence on $[Ca^{2+}]_i$ and their activity largely determine detrusor contractile state in human and animals (Brading, 2006; Kajioka et al., 2002; Hashitani et al., 2004b; Kirschstein et al., 2014). For example, entry of Ca^{2+} through L-type Ca^{2+} channels plays important roles in both human and rat bladder contraction (Fleichman et al. 2004; Schneider et al. 2004a, b).

In the human detrusor, carbachol-induced contraction has been associated with phosphoinositide hydrolysis (Harriss et al., 1995). However, the phospholipase C inhibitor U73122 did not significantly affect carbachol-stimulated bladder contraction, despite blocking inositol 1, 4, 5-tris-phosphate generation in humans and rats (Schneider et al., 2004a, b; Frazier et al., 2007). Schneider et al., (2004a) concluded that carbachol-induced contraction of the human urinary bladder via M_3 mAChR largely depends on Ca^{2+} entry through nifedipine-sensitive channels.

T-type Ca^{2+} channels activity has also been recorded in human, guinea-pig and rat detrusor myocytes (Igawa et al., 2014; Li et al., 2007; Sui et al., 2001; 2003) as well as interstitial cells of the bladder (Deng et al., 2012). T-type Ca^{2+} channels have roles in generating spontaneous excitation in the guinea-pig detrusor (Yanai et al., 2006). Ca^{2+} influx via T-type Ca^{2+}-channels mediates depolarizations that lead to action potentials and are coupled functionally to small-conductance Ca^{2+}-activated K^+ channels, contributing to the stability of the resting membrane potential in detrusor smooth muscle (Yanai et al., 2006). Yanai et al., (2006) thereby suggested that the pharmacological manipulation of T-type Ca^{2+} channels in detrusor smooth muscles could be of potential value for treating the overactive bladder. Ca^{2+}release from the sarcoplasmic reticulum seems to contribute to some contractile responses, for example, spontaneous vasoconstriction of suburothelial venules in the rat bladder appears to result from Ca^{2+}release from the sarcoplasmic reticulum upon activation of inositol 1, 4, 5-tris-phosphate receptors (Hashitani et al., 2011).

Less research has been done in the urethra compared to the bladder. In the urethra, both L-type and T-type Ca^{2+}-currents (Bradley et al., 2004) have been recorded in isolated urethral myocytes (Hollywood et al., 2003a) and rat urethral tissues (Shafei et al., 2003). L-type Ca^{2+} channels have the greatest influence on $[Ca^{2+}]_i$ and their activity largely determine the urethral tissue contractile state in human and animals (Brading, 2006; Bradley et al., 2004; Kajioka et al., 2002). Bradley et al., (2004) showed that the frequency of Ca^{2+} bursts in rabbit urethral smooth muscle was attenuated by blockade of T-type Ca^{2+} channels current. Thus, the L-type Ca^{2+} channels modulate urethra tonic contraction while the T-type Ca^{2+} channels modulate spontaneous activity.

5.3 CALCIUM SENSITIZATION IN THE LOWER URINARY TRACT

Rho kinase is expressed in the human detrusor (Kirschstein et al., 2015) with Rho kinase II showing a significantly greater expression than Rho kinase I. Ca^{2+} sensitization contributes to receptor-mediated contractile responses in the guinea-pig (Shahab et al., 2012b), pig (Tatsumiya et al., 2009), mouse (Chen et al., 2015; Isotani et al., 2004) and human (Kirschstein et al., 2014) detrusor smooth muscle. Fasudil (an inhibitor of Rho kinase) provokes relaxation of the pig bladder detrusor via both urothelium-dependent and independent pathways (Tatsumiya et al., 2009). Nakanishi et al., (2009) showed that the concentration of RhoA mRNA and activated RhoA were greater in urothelium than in pig bladder detrusor. Also, Y-27632 (an inhibitor of Rho kinase) showed a greater inhibitory effect in detrusor with intact urothelium than in that without urothelium (Nakanishi et al., 2009).

Chen et al., (2015) investigated the contribution of Thr694 and Thr852 phosphorylation sites in bladder smooth muscles from MYPT1 mutant mouse, by establishing two single point mutation mouse lines, T694A and T852A. MYPT1 was constitutively phosphorylated at T694 by unidentified kinases in vivo, whereas the Rho kinase phosphorylated the T852 site. They showed that phosphorylation of MYPT1 Thr694, but not Thr852, was the primary mechanism contributing to inhibition of myosin light chain phosphatase activity and enhancement of myosin light chain phosphorylation in vivo. Moreover, the constitutive

phosphorylation of MYPT1 Thr694 provided a mechanism for regulating force maintenance of the detrusor smooth muscle (Chen et al., 2015).

In detrusor smooth muscle, Ca^{2+} sensitization is modulated by Ca^{2+} mobilisation and vice versa. For example, Durlu-Kandilci & Brading, (2006) demonstrated that inhibitors of sarcoplasmic reticulum-released Ca^{2+} reduced carbachol-induced Ca^{2+} sensitization in the rat bladder. Moreover, Kirschstein et al., (2014) showed that human detrusor smooth muscle cells have two major intracellular mechanisms underlying mAChR-mediated contractions; activation of myosin light chain kinase predominantly following Ca^{2+}-influx via L-type Ca^{2+} channels, and activation of Rho kinase that is independent of a rise in intracellular Ca^{2+}, but occurred as a consequence of L-type Ca^{2+}channels activation (Kirschstein et al., 2014). On the other hand, constitutive Rho kinase activity was shown to be required for a quick stretch to activate Ca^{2+} entry and cause a myogenic contraction of the detrusor (Poley et al., 2008).

Recently, Tsai et al., (2014) showed that MYPT1 knockout did not cause significant derangement of bladder or ileal smooth muscle contractility in mice (Gao et al., 2016). Thus, the results suggest that in these tissues, Ca^{2+} sensitization is dependent on myosin light chain kinase and myosin light chain phosphatase activities without changes in constitutive MYPT1 phosphorylation. Also, PKC contributes to receptor-mediated Ca^{2+} sensitization in the rat and guinea-pig bladder (Durlu-Kandilci & Brading, 2006). However, animal studies suggested that PKC plays a minimal role in bladder contractility (Durlu-Kandilci & Brading, 2006; Fleichman et al., 2004; Schneider et al., 2004a).

Again less research has been carried out on the urethra compared to the bladder. RhoA is expressed in the rat urethra (Teixeira et al., 2007). Moreover, the urethra expresses other Ca^{2+} sensitization elements; Rho kinase I and Rho kinase II. However, Rho kinase I and Rho kinase II protein levels were significantly lower in the rat urethral tissues compared to the trigone and the detrusor (Teixeira et al., 2007). Incubation with a Rho kinase inhibitor inhibited contractions elicited by phenylephrine in the rabbit urethra (Walsh et al., 2011). Also, a role for RhoA and Rho kinase in urethral tone was indicated by the demonstration that inhibition of RhoA with Clostridium difficile toxin B, or inhibition of Rho kinase with Y27632, abolished porcine urethral tone without affecting intracellular Ca^{2+} (Malmqvist et al.,

2004). These results suggest that Rho kinase plays a major role in urethral smooth muscle contraction (Walsh et al., 2011).

5.4 CALCIUM CHANNELS AND CALCIUM SENSITISATION RELATED PATHOPHYSIOLOGY IN THE LOWER URINARY TRACT

L-type and T-type Ca^{2+} channels can be affected by pathological conditions. The density of T-type channels Ca^{2+} current is increased, and that of L-type Ca^{2+} decreased, in human detrusor myocytes from idiopathic detrusor overactivity patients compared to normal bladders (Sui et al., 2007). However, total Ca^{2+} current density was similar. A greater T-type Ca^{2+}-channels density was also recorded in isolated myocytes from bladder outlet obstruction and overactive bladder rat models (Li et al., 2007). Also, reports have shown that enhanced extracellular Ca^{2+} influx through L-type Ca^{2+} channels mediate overactive detrusor in diabetic mouse (Leiria et al., 2011). Thus, an increase in the density of L-type Ca^{2+} channels which may enhance extracellular Ca^{2+} influx seems to contribute to conditions such as the overactive bladder.

Ca^{2+} sensitization is thought to contribute to pathological conditions underlying benign prostatic hyperplasia, erectile dysfunction, kidney failure, bladder outlet obstruction (Boopathi et al., 2014; Shahab et al., 2012a), ejaculation disorders, prostate cancer, bladder cancer initiation and metastasis (Gur et al., 2011). Takahashi et al., (2009) showed enhancement of the mAChR-coupled RhoA/Rho kinase pathway in the detrusor of rats with bladder outlet obstruction. Rho kinase enhanced signalling may mediate a compensatory mechanism for expelling urine against the obstruction (Shahab et al., 2012a; Takahashi et al., 2009). The association of Ca^{2+} sensitization with physiology and pathophysiology of smooth muscle contraction suggests that a greater understanding of the Ca^{2+}-sensitization pathway might provide a novel drug target for stress urinary incontinence.

The overall aim of this study, therefore, was to gain further insights into the intracellular signalling pathways involved in the regulation of porcine urethral smooth muscle contraction. This study investigated the classical Ca^{2+} pathway, as well as Ca^{2+} sensitization for both basal

urethral tone and α_1-AR-mediated contractions. The following specific questions were addressed:

- Which signalling pathways mediate maintenance of urethral intrinsic basal tone?
- What are the intracellular pathways associated with α_1-AR-mediated responses?
- Does agonist concentration determine which intracellular pathways contribute to contraction?

5.5 MATERIALS AND METHODS

Methods

Strips of old female pig urethra from the proximal region (with urothelium/LP) were set up in 8ml organ baths in pairs as described in chapter 2 (as control and test tissues). Following set up and equilibration for 1 hour, the effects of inhibitors of intracellular signalling molecules were investigated. These included fasudil (10μM), Y27632 (10μM), nifedipine (100nM), cyclopiazonic acid (CPA) (10μM), indomethacin (10μM) and calphostin C (1μM). These drugs inhibited Rho kinase, Ca^{2+} influx via L-type Ca^{2+} channels, Ca^{2+} release via sarcoplasmic reticulum, cyclooxygenase and PKC respectively (Figure 5.1). Incubating tissues in Krebs solution without Ca^{2+} and with EDTA (3mM) was also used to block extracellular Ca^{2+} influx. The tissue pairs were incubated with/without the inhibitor for 30 minutes after equilibration to investigate the contribution of intracellular pathways to basal tone and α_1-AR-mediated responses.

Changes in basal tone were measured after 30 minutes incubation with an inhibitor to investigate the contribution of intracellular signalling molecules to the basal tone, and basal tone was compared between tissues incubated with and without inhibitors. Following this cumulative concentration-response curves to noradrenaline, phenylephrine and A61603 were constructed and maximum responses or sensitivity pEC_{50} values calculated as described in chapter 2. The contribution of intracellular signalling molecules to equivalent, half maximal, three-quarter maximal and maximal responses to agonists was also investigated using single agonist concentrations shown in Table 5.1. These concentrations were extrapolated from the cumulative concentration curves for agonists as illustrated in Figure 5.2.

In all experiments, only one drug was tested on each tissue. The α_1-AR-mediated responses were analysed and data expressed as described in chapter 2.

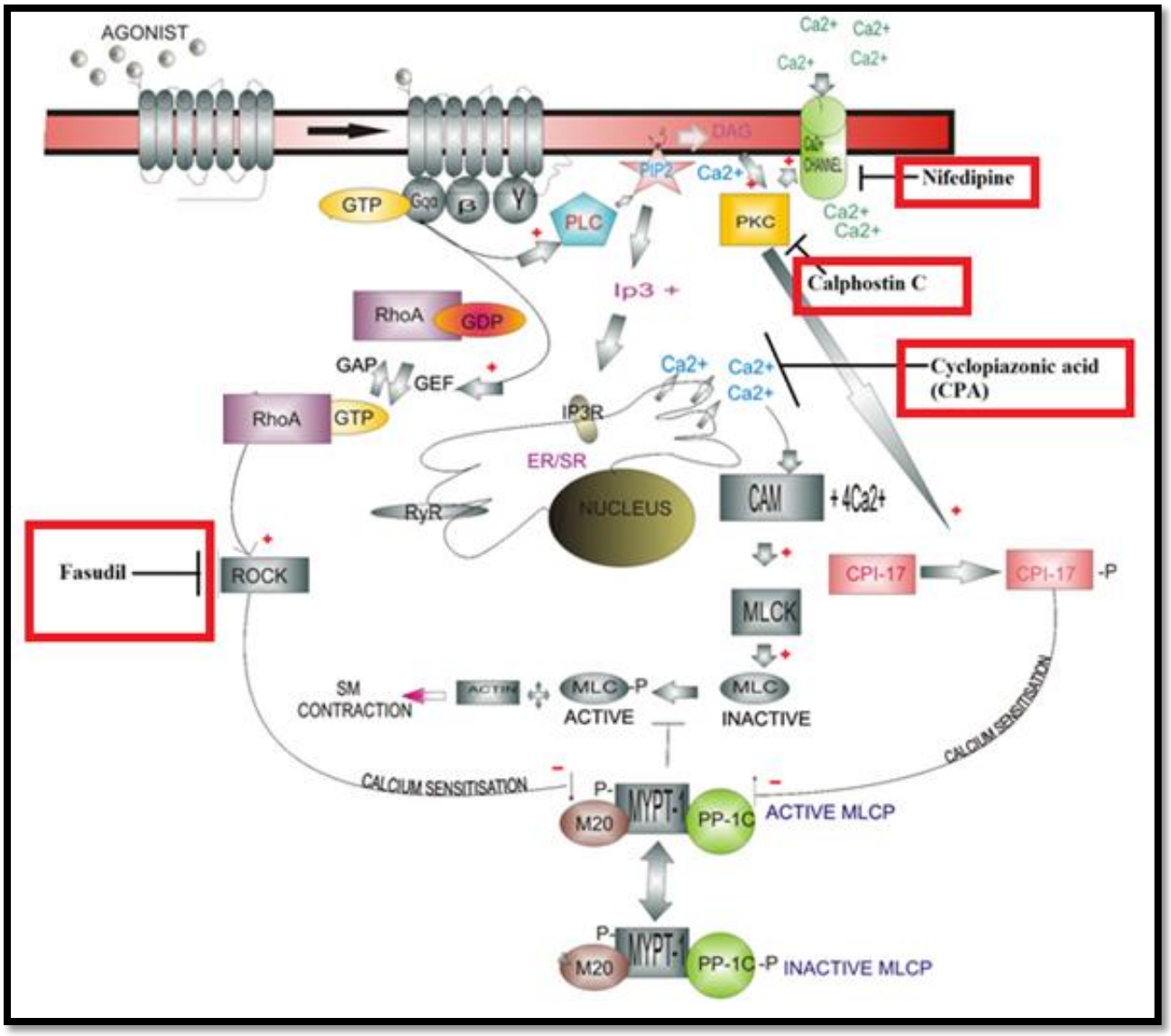


Figure 5.1 Intracellular signalling pathways inhibited to allow analysis of pathways activated by α_1-adrenoceptor activation. Fasudil blocks Rho kinase (ROCK), nifedipine blocks Ca^{2+} influx via the L-type Ca^{2+} channels, calphostin C blocks protein kinase C whilst cyclopiazonic acid blocks sarcoplasmic reticulum release of Ca^{2+}.

	Concentrations inducing equivalent response	*Concentrations inducing half maximal response*	*Concentrations inducing three-quarter maximal response*	*Concentrations inducing maximal response*
Noradrenaline	10μM	4μM	10μM	1mM
Phenylephrine	10μM	5μM	10μM	1mM
A61603	30nM	37nM	300nM	100μM

Table 5.1 The concentrations of agonists producing equivalent, half maximal, three-quarter maximal and maximal responses, chosen to compare the contribution and role of intracellular signalling pathways in urethral smooth muscle.

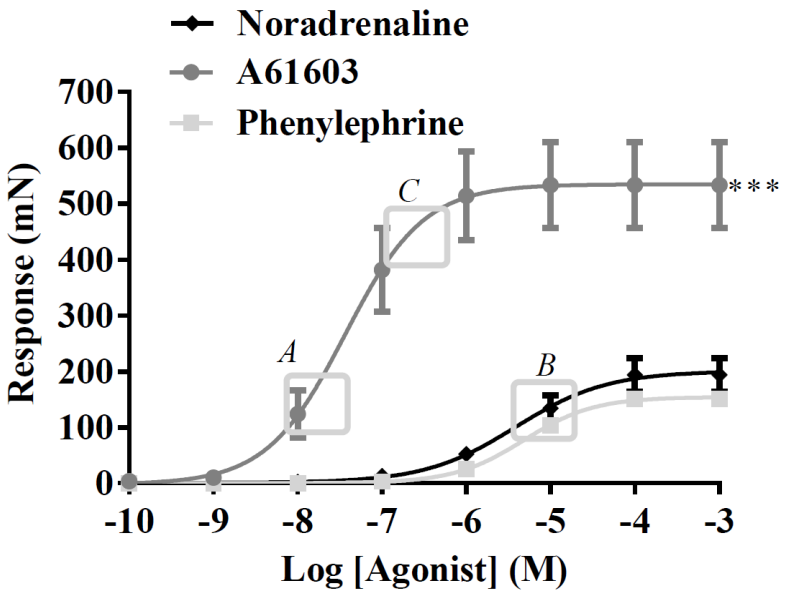


Figure 5.2 Concentration-response curves to phenylephrine, noradrenaline and A61603 in porcine proximal urethral tissues, showing choice of concentrations of agonists inducing equivalent, half maximal, three-quarter of maximal and maximal responses. Point A and B corresponded to where contractile responses to noradrenaline, phenylephrine and A61603 were equivalent and the concentration that induced these responses were 10μM, 10μM, and 30nM of noradrenaline, phenylephrine and A61603 respectively. Point B also coincidentally was the three-quarter of maximal responses of noradrenaline and phenylephrine; therefore point B(s) were also compared to point C, which was the three-quarter of maximal responses of A61603. Furthermore, half maximal and maximal responses induced by agonists were also compared. n=7-8; *** P<0. 001 vs. maximum contractile response to noradrenaline (One-way ANOVA with Tukey post-hoc test).

5.6 RESULTS

The intracellular pathways contributing to urethral basal tone

The basal tone of the urethral tissues consistently increased slowly over time during the equilibration period (Figure 5.3) and plateaued after 40-60 minutes.

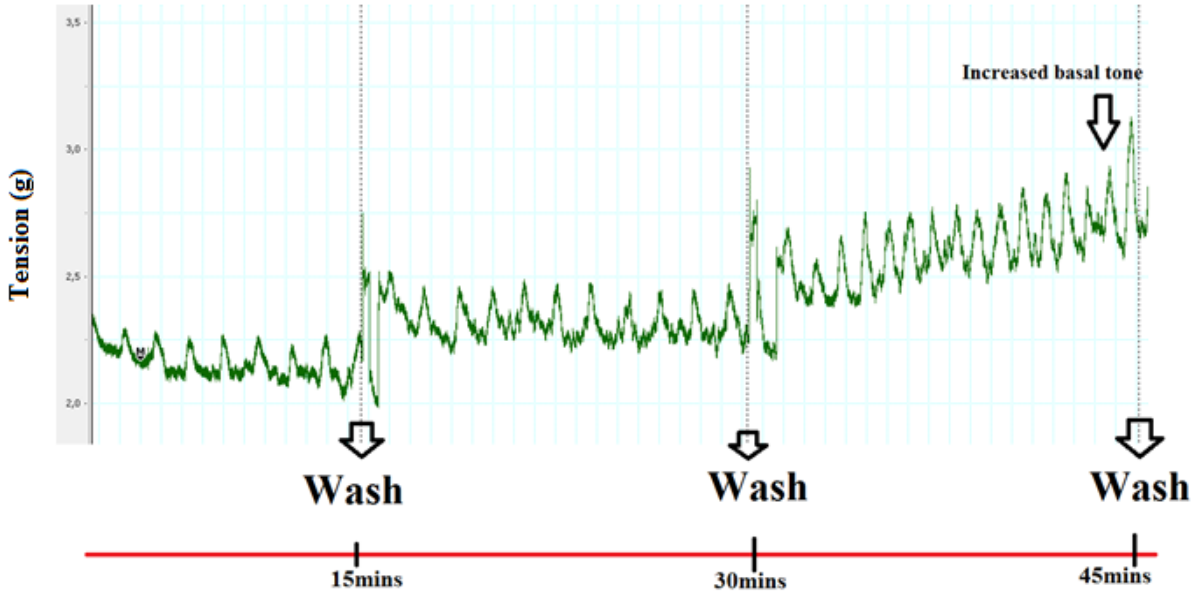


Figure 5.3 Example of spontaneous development of porcine urethral basal tone during the equilibration period (1g= 100mN).

Incubation with Y27632, fasudil, nifedipine, indomethacin or Ca^{2+} exclusion/EDTA significantly reduced the basal tone of the urethral strips (Table **5.2**; Figure 5.4). Nifedipine reduced basal tone by 155.1±17.7% ($p<0.001$), whilst Ca^{2+} exclusion/EDTA reduced basal tone by 169.2±19.6% ($p<0.0001$). Fasudil and Y27632 significantly reduced the basal tone of the urethra by 357.2±31.2% ($p<0.0001$) and 688.7±45.8% ($p<0.0001$) respectively. Moreover, indomethacin (10µM) reduced urethra tissue basal tone by 219.3±37.6% ($p<0.001$). However, no change in the urethral basal tone was observed following incubation with CPA or calphostin C.

	Control (mN)	Test (mN)	Percentage reduction (%)	n
+ Nifedipine (100nM)	26.5±8.3	**-14.6±4.7*** **	155.1±17.7	10
Ca^{2+} exclusion/EDTA (3mM)	29.3±5.7	**-20.3±5.7**** **	169.2±19.6	11
+ CPA (10µM)	27.5±5.9	16.5±11.5	40.0±41.7	10
Indomethacin (10µM)	28.7±6.1	**-34.3±10.8*** **	219.3±37.6	7
Fasudil (10µM)	29.0±5.9	**-74.5±9.0**** **	**357.2±31.2+++**	17
Y27632 (10µM)	26.2±7.8	**-154.1±12.0**** **	**688.7±45.8+++**	7
+ Cal C (1µM)	25.2±7.0	31.0±8.0	**+23.2±31.9++**	10

Table 5.2 Mean (±SEM) basal urethral tone (proximal tissues with urothelium/lamina propria), with or without inhibitor. ***P<0.001 and ****P<0.0001 compared to the control responses (Student's t-test), (++p<0.01, +++p<0.001 vs. percentage reduction in maximum for Ca^{2+} exclusion/EDTA (3mM) (One-way ANOVA with Tukey post-hoc test). Negative values= tone went below initial basal tone; positive value= tone of test tissue was greater than control tissue.

Intracellular signalling contributing to α_1-adrenoceptor-mediated urethral contractile responses of urethra

Noradrenaline: Incubation with the various inhibitors caused a significant decrease in the contractile responses to cumulative concentrations of noradrenaline (Table 5.3; Figure 5.5).

	Control Max (mN)	Test Max (mN)	Percentage reduction in max response (%)	Control pEC_{50}	Test pEC_{50}	n
Nifedipine	198.2±31.7	**92.9±14.4****	53.1±7.3	5.4±0.2	5.2±0.2	8
Ca^{2+} free/EDTA(3mM)	211.9±33.0	**24.8±5.6**** **	**88.3±2.6++++**	5.5±0.2	4.8±0.3	7
CPA	240.8±15.3	**162.9±23.1***	32.4±9.6	5.3±0.1	5.4±0.2	7
Fasudil	285.1±29.5	**140.5±12.5*****	50.7±4.4	5.2±0.1	4.9±0.1	8
Calphostin C	243.6±18.3	**167.7±14.1****	31.2±5.8	5.3±0.1	5.2±0.1	6

Table 5.3 Mean (±SEM) maximum contractile responses and pEC_{50} values for noradrenaline in the presence or absence of inhibitors (proximal tissues with urothelium/lamina propria). *p<0.05, **p<0.01, *** P<0.001, **** P<0.0001 vs. respective control responses (Student's t-test). (++++P<0.0001 (one-way ANOVA with Bonferroni post-hoc test)).

The reduction in maximum contractile responses was greatest in tissues incubated in Ca^{2+} free/EDTA solution ($p<0.0001$). The pEC_{50} values for noradrenaline for urethral tissues incubated with or without inhibitors were similar and unchanged by any of the inhibitors.

Similar to responses observed with noradrenaline, contractile responses to phenylephrine were significantly decreased after incubation with nifedipine, Ca^{2+} free solution /EDTA, CPA and fasudil (Table 5.4, Figure 5.6). Reductions in maximum contractile responses were greatest in tissues incubated with CPA (10µM). However, a non-significant potentiation of the responses to phenylephrine was observed in tissues incubated with calphostin C (1µM) as shown in Figure 5.6E. Furthermore, pEC_{50} values for phenylephrine for tissues incubated with or without inhibitors were unchanged by any of the inhibitors.

	Control response (mN)	Test response (mN)	Percentage reduction in Max (%)	Control pEC_{50}	Test pEC_{50}	n
Nifedipine	150.5±6.2	**67.8±6.3******	54.9±4.2	5.3±0.1	5.1±0.1	8
Ca^{2+} free/EDTA(3mM)	150.5±5.6	**73.2±7.0******	51.4±4.6	5.4±0.1	5.1±0.1	8
CPA	180.2±22.0	**74.8±15.6****	58.5±8.7	5.4±0.2	5.4±0.2	8
Fasudil	154.7±6.5	**103.3±16.2***	33.2±10.5	5.3±0.1	5.0±0.1	6
Calphostin C	155.1±6.5	**209.8±28.0**	+35.2±18.1	5.3±0.1	5.4±0.2	6

Table 5.4 Mean (±SEM) maximum contractile responses and pEC_{50} values for phenylephrine with or without signalling inhibitors (proximal tissues with urothelium/lamina propria). *$p<0.05$, **$p<0.01$, and **** $P<0.0001$ vs. respective control responses (Student's t-test). Positive value= maximum response of test tissue was greater than control tissue.

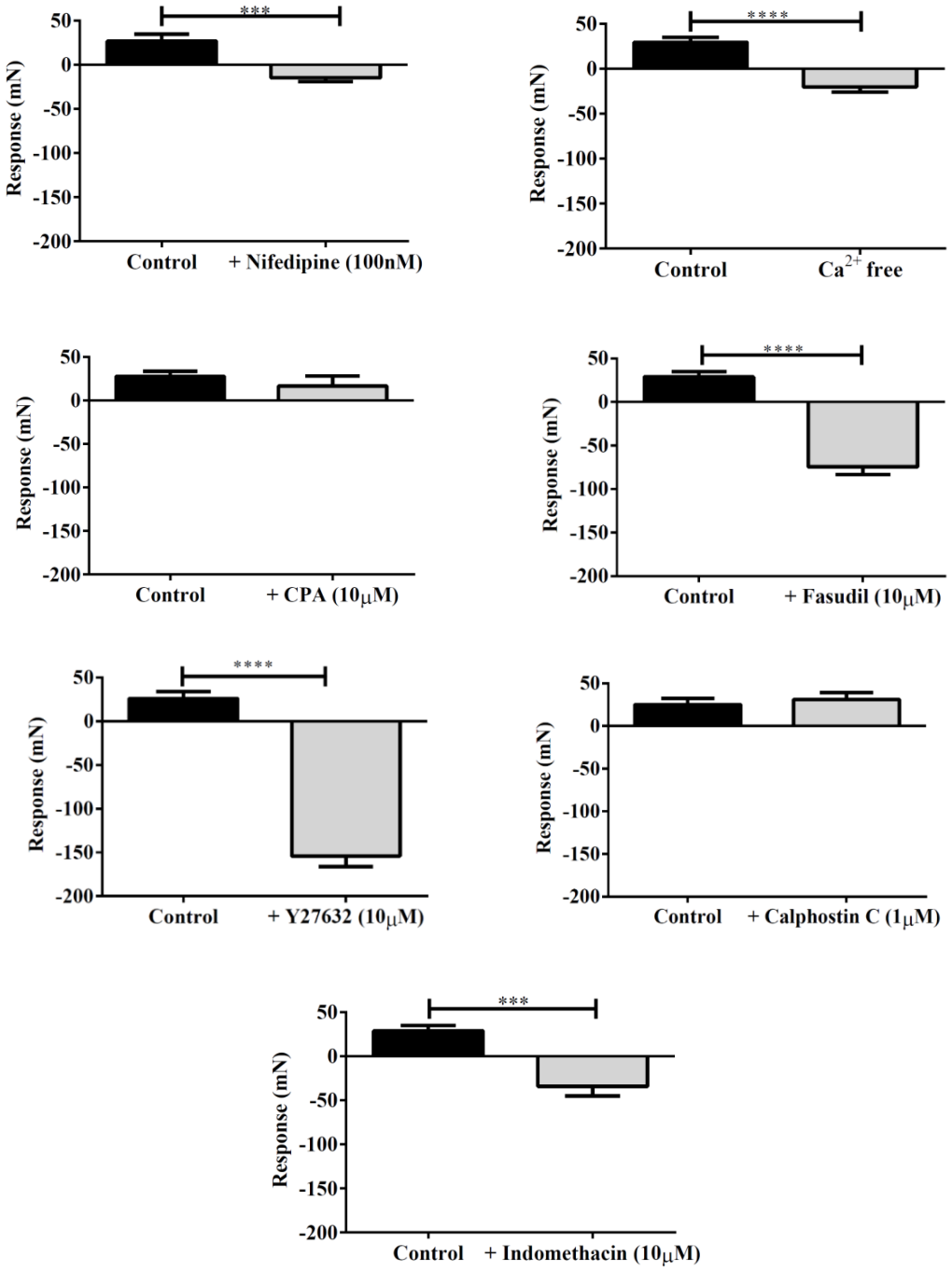


Figure 5.4 Mean (±SEM) urethral basal tone with or without inhibitors of signalling pathways (proximal tissues with urothelium/lamina propria). n=7-17; ***P<0.001 and ****P<0.0001 compared to the control tissues (Student's t-test).

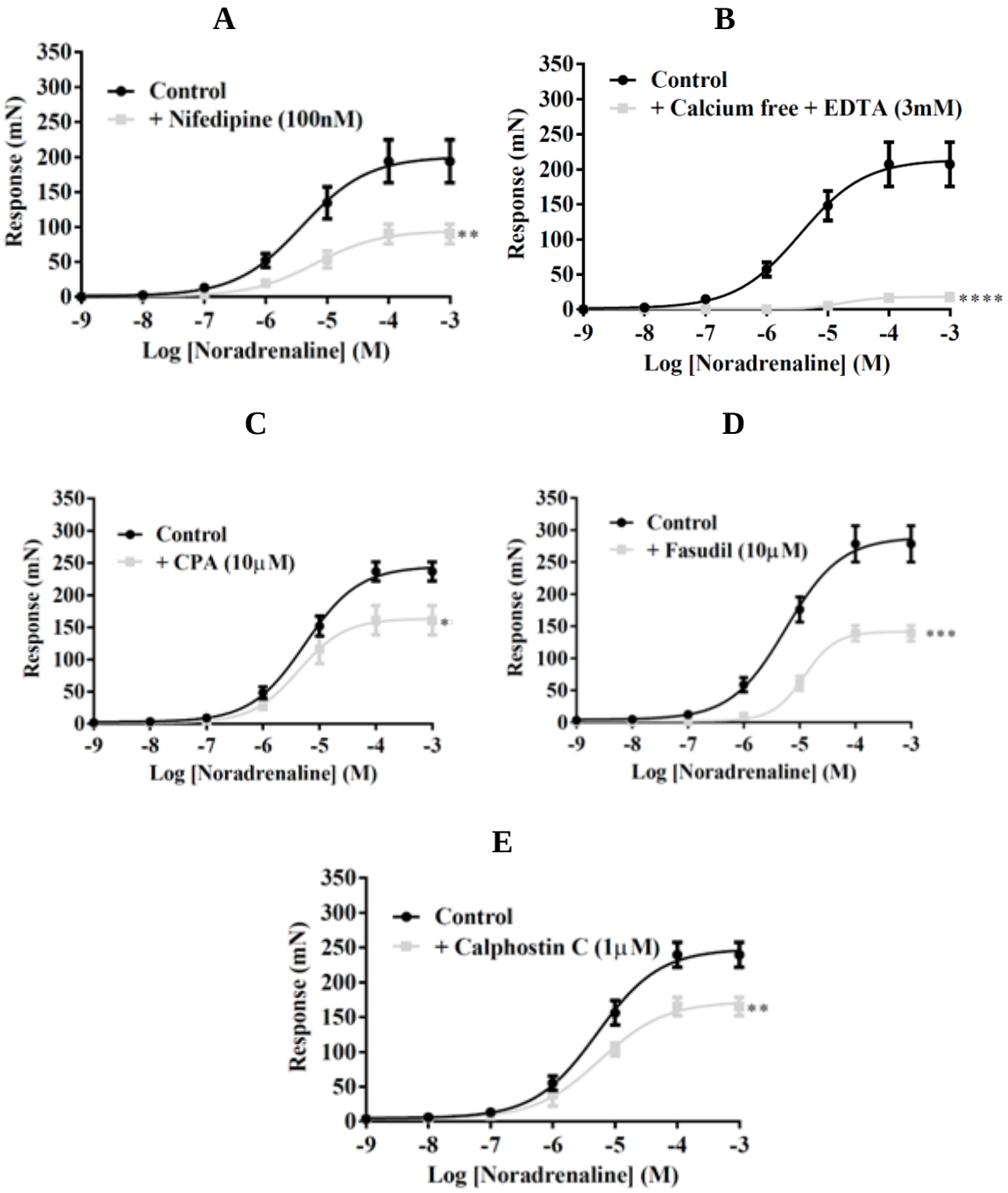


Figure 5.5 Mean (±SEM) concentration-response curves to noradrenaline with or without inhibitors of extracellular Ca^{2+} influx (A-B), intracellular Ca^{2+} release (C), Rho kinase (D) and protein kinase C (E) (proximal tissues with urothelium/lamina propria). n=6-8; *p<0.05, **p<0.01, *** P<0.001, **** P<0.0001 vs. control responses (Student's t-test).

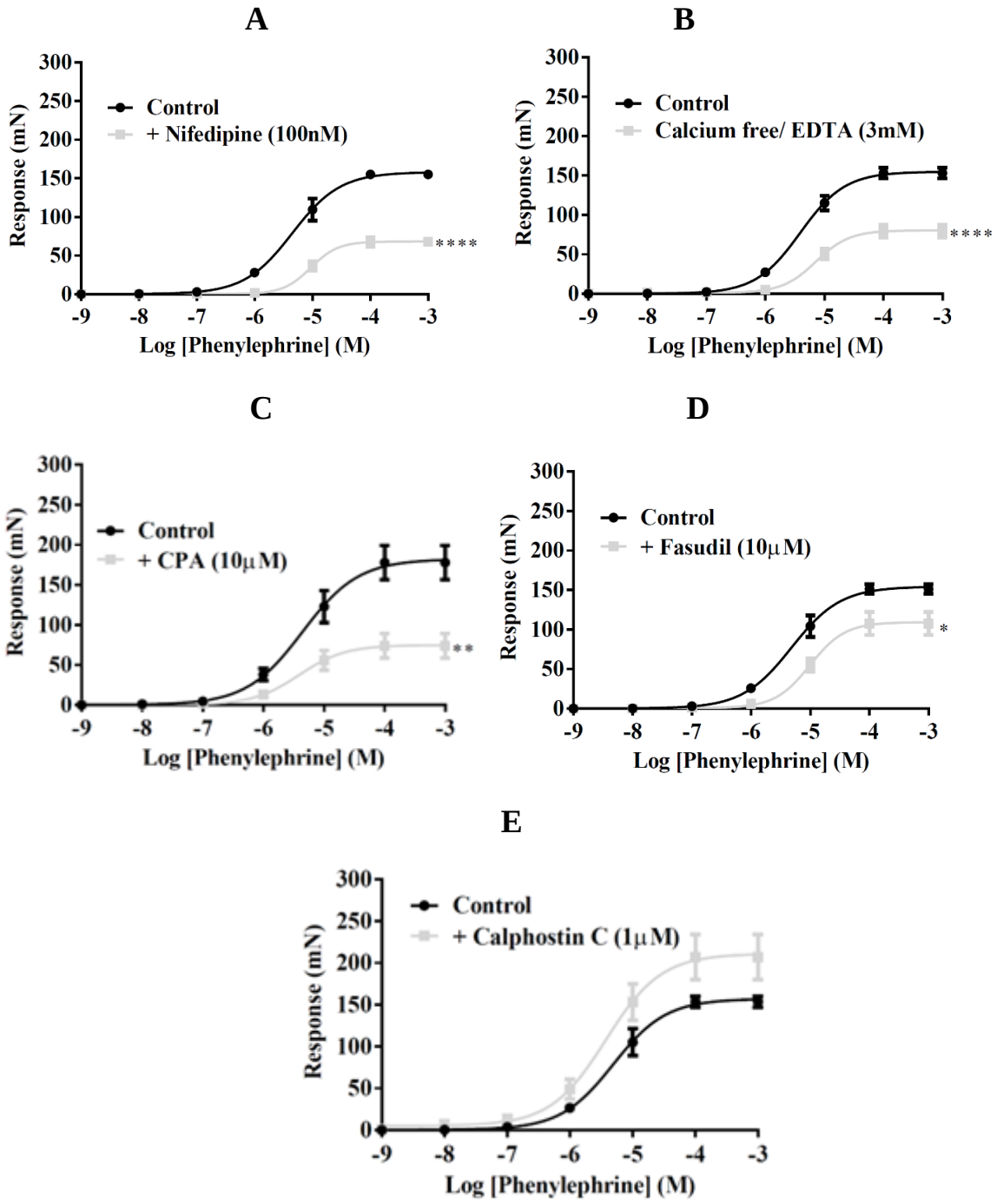


Figure 5.6 Mean (±SEM) concentration-response curves to phenylephrine in the presence or absence of inhibitor of extracellular Ca^{2+} influx (A-B), intracellular Ca^{2+} release (C), Rho kinase (D) and protein kinase C (E) (proximal tissues with urothelium/lamina propria). n=6-8. *p<0.05, ** P<0. 01, **** P<0.0001 vs. control responses (Student's t- test)

Responses to A61603 were also significantly decreased after prior incubation with inhibitors (Table 5.5, Figure 5.7), with the greatest inhibition in tissues incubated with calphostin C ($p<0.001$). The pEC_{50} values for A61603 for tissues incubated with or without Ca^{2+} free solution /EDTA, CPA, fasudil and calphostin C were similar. In contrast, pEC_{50} values for A61603 for tissues incubated with nifedipine were significantly lower compared to those without nifedipine ($p<0.05$). The concentration-response curves of tissues incubated with nifedipine were shifted to the right significantly, compared to those without nifedipine.

	Control response (mN)	**Test response (mN)**	**Percentage reduction in Max (%)**	**Control pEC_{50}**	**Test pEC_{50}**	**n**
Nifedipine	576.4±75.3	**302.2±61.0***	47.6±10.6	7.5±0.2	**6.8±0.2***	6
Ca^{2+} free/EDTA(3mM)	531.2±90.6	**204.2±24.6****	61.5±4.6	7.5±0.3	7.1±0.1	6
CPA	505.3±66.6	**204.0±24.6*****	59.6±4.9	7.6±0.2	7.4±0.2	8
Fasudil	533.7±76.6	**290.3±28.1***	45.6±5.3	7.4±0.2	7.2±0.1	7
Calphostin C	566.9±81.7	**83.9±22.9*****	**85.2±4.0++**	7.4±0.2	6.3±0.6	6

Table 5.5 Mean (±SEM) urethral maximum contractile responses, pEC_{50} values and percentage reduction in maximum response for A61603 in the presence of inhibitors (proximal tissues with urothelium/lamina propria). *$p<0.05$, ** $P<0.01$, *** $P<0.001$ vs. respective control responses (Student's t-test). (++$p<0.01$ (One-way ANOVA with Bonferroni post hoc-test)).

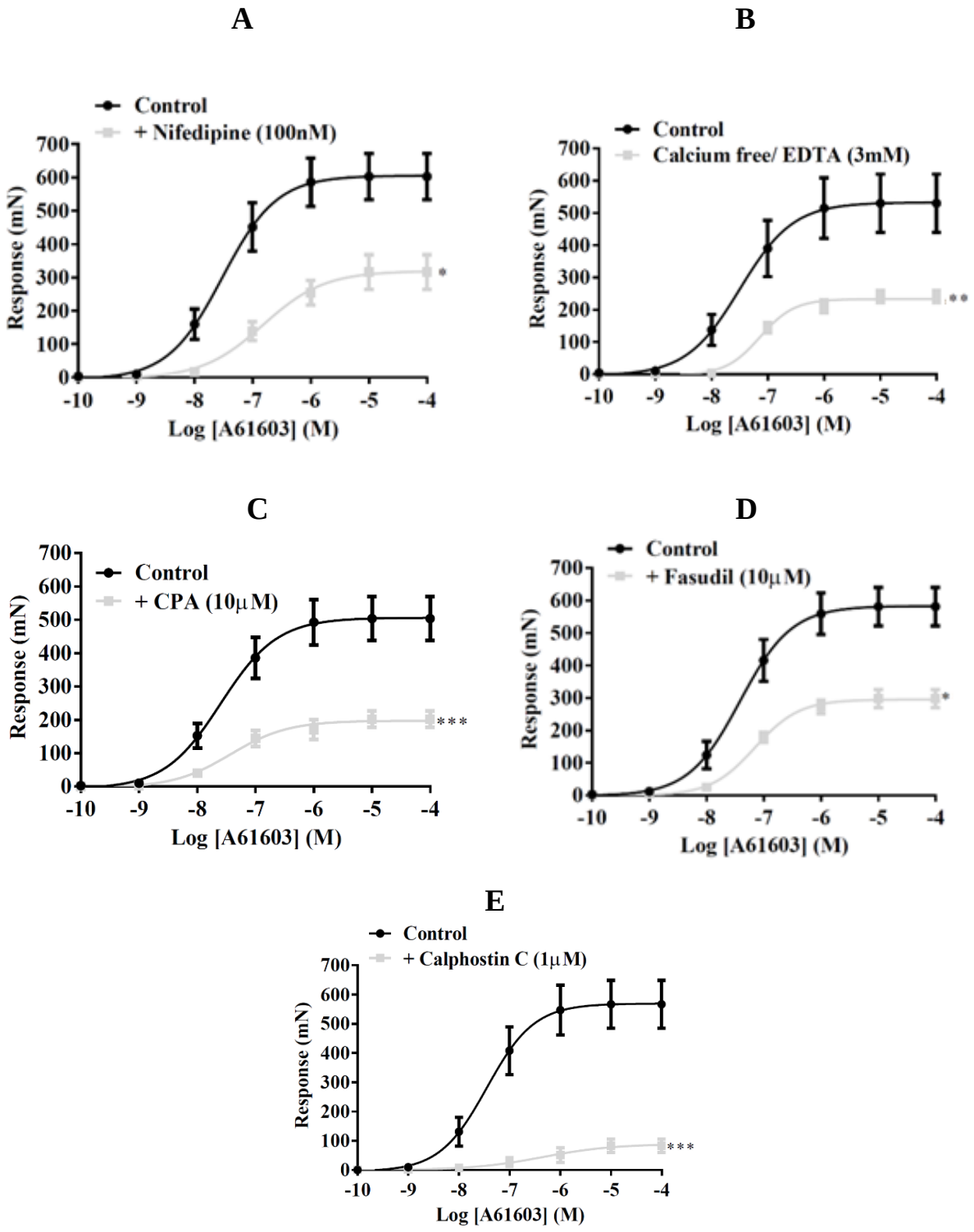


Figure 5.7 Mean (±SEM) concentration-response curves to A61603 for urethral tissues with or without an inhibitor of extracellular Ca^{2+} influx (A-B), intracellular Ca^{2+} release (C), Rho kinase (D) and protein kinase C (E) (proximal tissues with urothelium/lamina propria). n=6-8; *p<0.05, ** P<0. 01, *** P<0.001 vs. maximum contractile responses of the control tissues (Student's t-test).

Intracellular signalling involved in producing an equivalent, half maximal, three-quarter maximal and maximal response in urethra

For comparison of the contributions of the intracellular signalling pathways involved in producing an equivalent, half maximal, three-quarter maximal and maximal response, three points were chosen from concentration curves for control tissues (as shown in Figure 5.2).

Signalling pathways involved in producing maximal responses

Maximal contractile responses to noradrenaline were reduced significantly by all inhibitors (Table 5.6A; Figure 5.8, Figure 5.9). Ca^{2+} exclusion /EDTA had the greatest inhibitory effect on noradrenaline-induced maximal contraction while calphostin C (1µM) had the lowest inhibitory effect.

All inhibitors reduced the maximal response to phenylephrine (1mM) except for calphostin C (1µM) (Table 5.6B; Figure 5.8. Figure 5.9), which produced a non-significant potentiation. Lastly, maximum responses to A61603 were reduced by all inhibitors (Table 5.6C; Figure 5.8. Figure 5.9). The inhibitory effect was greatest with calphostin C (1µM) ($p<0.001$).

(A)Noradrenaline	+ Nifedipine		Ca^{2+} free/EDTA		+ CPA		+ Fasudil		+ Calphostin C	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Response (mN)	198.2±31.7	**92.9±14.4****	211.9±33.0	**24.8±5.6 ******	240.8±15.3	**162.9±23.1***	285.1±29.5	**140.5±12.5 *** **	243.6±18.3	**167.7±14.1****
Percentage reduction in Max (%)	53.1±7.3		**88.3±2.6++++**		32.4±9.6		50.7±4.4		31.2±5.8	
n	8		7		7		8		6	

(B) Phenylephrine	+ Nifedipine		Ca^{2+} free/EDTA		+ CPA		+ Fasudil		+ Calphostin C	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Response (mN)	150.5±6.2	**67.8±6.3 ******	150.5±5.6	**73.2.±7.0 ******	180.2±22.0	**74.8±15.6****	154.7±6.5	**103.3±16.2***	155.1±6.5	209.8±28.0
Percentage reduction in Max (%)	54.9±4.2		51.4±4.6		58.5±8.7		33.2±10.5		+35.2±18.1	
n	8		8		8		6		6	

(C) A61603	+ Nifedipine		Ca^{2+} free/EDTA		+ CPA		+ Fasudil		+ Calphostin C	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Response (mN)	576.4±75.3	**302.2±61.0***	531.2±90.6	**204.2.±24.6 ** **	505.3±66.6	**204.0±24.6 *** **	533.7±76.6	**290.3±28.1***	566.9±81.7	**83.9±22.9*****
Percentage reduction in Max (%)	47.6±10.6		61.5±4.6		59.6±4.9		45.6±5.3		**85.2±4.0++**	
n	6		6		8		7		6	

Table 5.6 Mean (±SEM) maximal contractile responses and percentage reduction in maximum response to (A) noradrenaline, (B) phenylephrine and (C) A61603 with or without inhibitor (proximal tissues with urothelium/lamina propria). *p<0.05, ** P<0. 01, *** P<0. 001, ****p<0.0001 vs. control (Student's t-test). ++ P<0.01, ++++p<0.0001 (One-way ANOVA with Bonferroni post-hoc test). Positive value= maximum response of test tissue was greater than control tissue.

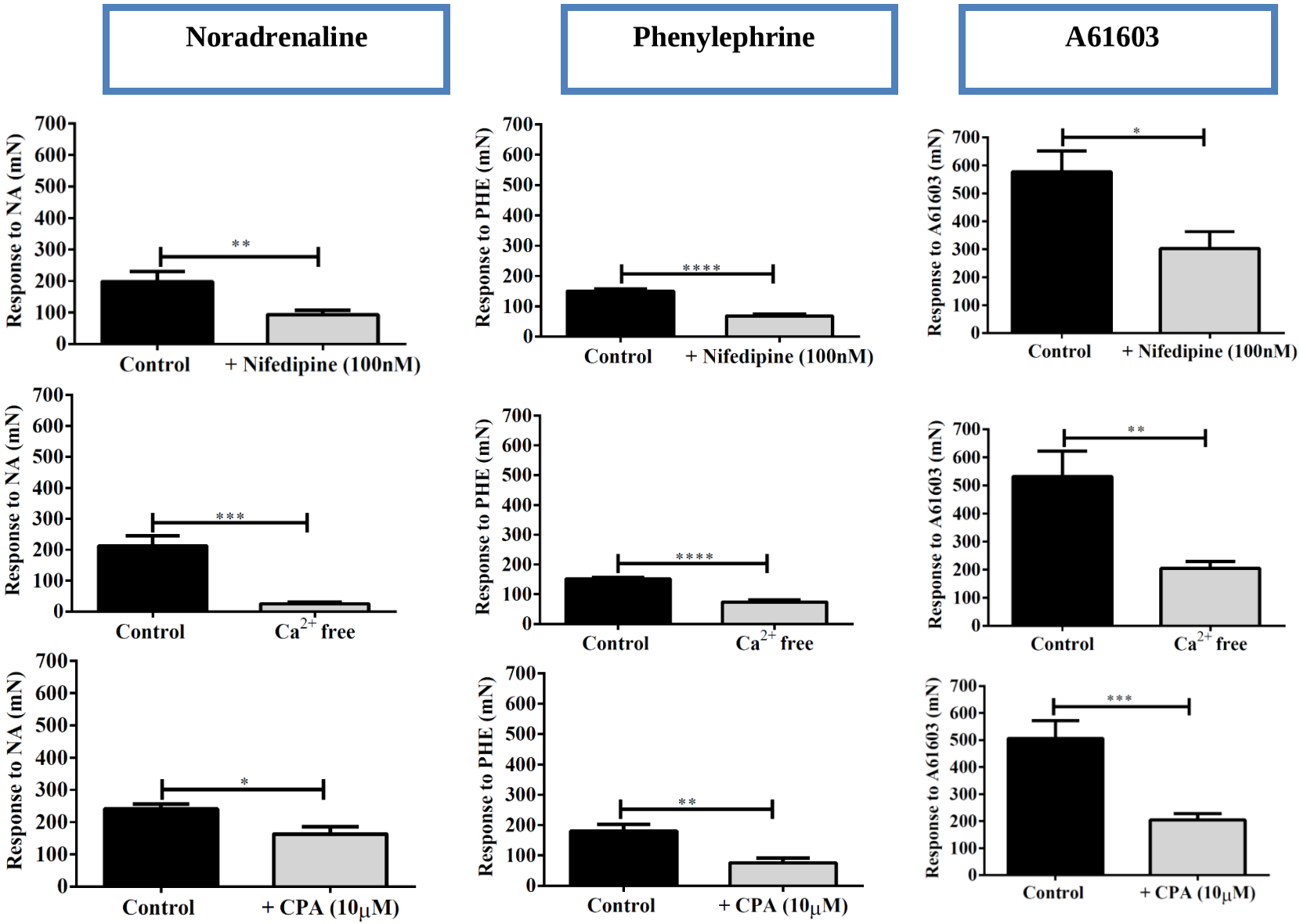


Figure 5.8 Mean (±SEM) maximal contractile responses to noradrenaline, phenylephrine and A61603 in the presence or absence of inhibitor blocking the L-type Ca^{2+} channel, Ca^{2+} influx from the extracellular space and sarcoplasmic reticulum Ca^{2+} release (proximal tissues with urothelium/lamina propria). n=6-8. *P<0.05, ** P<0.01, *** P<0.001, and ****p<0.0001 vs. control (Student's t-test). NA= noradrenaline, PHE = phenylephrine.

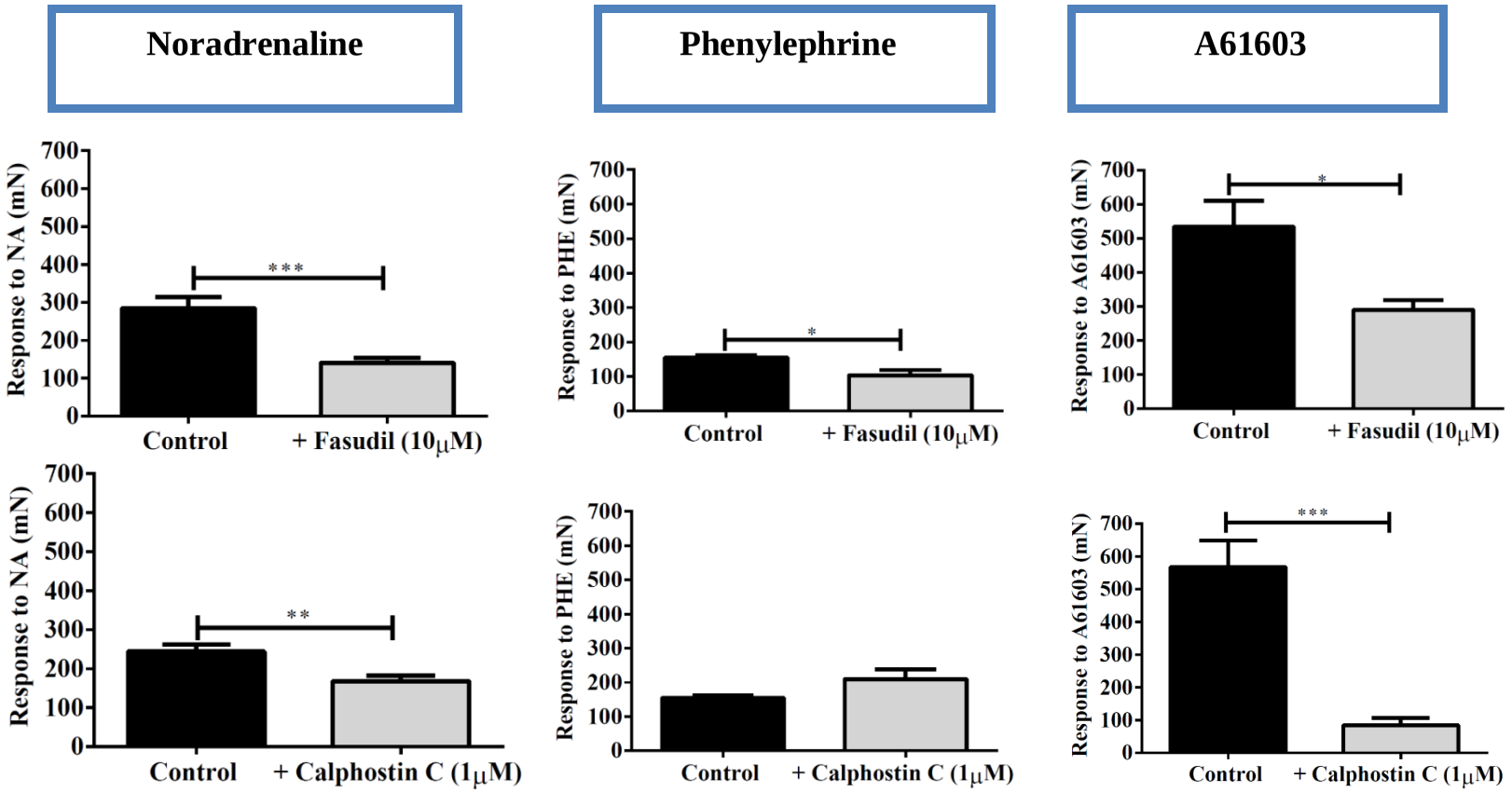


Figure 5.9 Mean (±SEM) maximal contractile responses to noradrenaline, phenylephrine and A61603 in the presence or absence of inhibitor blocking the Rho kinase and PKC (proximal tissues with urothelium/lamina propria). n=6-8. *P<0.05, ** P<0.01, and ***p<0.001 vs. control (Student's t-test). NA= noradrenaline, PHE = phenylephrine.

Signalling pathways involved in producing equivalent responses

Effect of inhibitors on equivalent responses induced by noradrenaline, phenylephrine or A61603 was also analysed.

Noradrenaline: Incubation with inhibitors reduced responses of noradrenaline (Table 5.7A; Figure 5.10, Figure 5.11). Exclusion of Ca^{2+} from the bathing solution had the greatest inhibitory effect on noradrenaline-induced contractions ($p<0.0001$). Incubation with nifedipine, fasudil and calphostin C also had an inhibitory effect on the responses to noradrenaline but not CPA.

Phenylephrine: In addition, contractile responses to phenylephrine were significantly reduced by nifedipine, Ca^{2+} free/EDTA, CPA and fasudil (Table 5.7B; Figure 5.10, Figure 5.11). However, incubation with calphostin C had no significant effect on tissue responses.

A61603: Also, contractile responses to A61603 were significantly reduced by all inhibitors (Table 5.7C; Figure 5.10, Figure 5.11). Incubation with calphostin C had the greatest inhibitory effect on responses ($p<0.01$), followed by Ca^{2+}free/EDTA ($p<0.05$), nifedipine ($p<0.05$), fasudil ($p<0.05$) and CPA ($p<0.05$).

(A)Noradrenaline	+ Nifedipine		Ca^{2+} free/EDTA		+ CPA		+ Fasudil		+ Calphostin C	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Response (mN)	137.5±23.4	**55.5±12.1****	151.2±22.0	**6.8.±2.4 ******	154.8±16.0	117.4±22.1	181.0±20.7	**61.6±11.3*****	160.2±17.8	**105.9±10.0***
Percentage reduction in Max (%)	53.4±7.3		**95.5±1.6++++**		24.1±14.3		66.0±6.3		33.9±6.2	
n	8		7		7		8		6	

(B) Phenylephrine	+ Nifedipine		Ca^{2+} free/EDTA		+ CPA		+ Fasudil		+ Calphostin C	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Response (mN)	117.0±8.0	**30.1±6.2 ******	115.8±7.0	**47.3.±5.6 ******	123.9±20.5	**55.5±12.7 ***	105.0±15.9	**51.5±9.3***	105.4±16.0	154.2±21.9
Percentage reduction in Max (%)	74.3±5.3		59.1±4.9		55.2±10.2		51.0±8.8		+46.4±20.8	
n	8		8		8		6		6	

(C) A61603	+ Nifedipine		Ca^{2+} free/EDTA		+ CPA		+ Fasudil		+ Calphostin C	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Response (mN)	100.2±27.1	**27.1±6.2***	96.3±27.7	**19.5.±1.6***	100.7±19.9	**37.5±8.9***	90.6±24.1	**30.0±2.0***	96.4±27.6	5.0±3.0**
Percentage reduction in Max (%)	73.7±6.6		80.7±2.6		63.1±8.5		65.7±3.3		**95.0±3.3++**	
n	6		6		8		7		6	

Table 5.7 Mean (±SEM) equivalent contractile responses of urethral tissues to noradrenaline, phenylephrine and A61603 with or without inhibitor (proximal tissues with urothelium/lamina propria). *P<0.05, ** P<0.01, ***p<0.001 and ****p<0.0001 vs. control (Student's t-test). ++p<0.01 and ++++p<0.0001 (One-way ANOVA with Bonferroni post-hoc test). Positive value= response of test tissue was greater than control tissue.

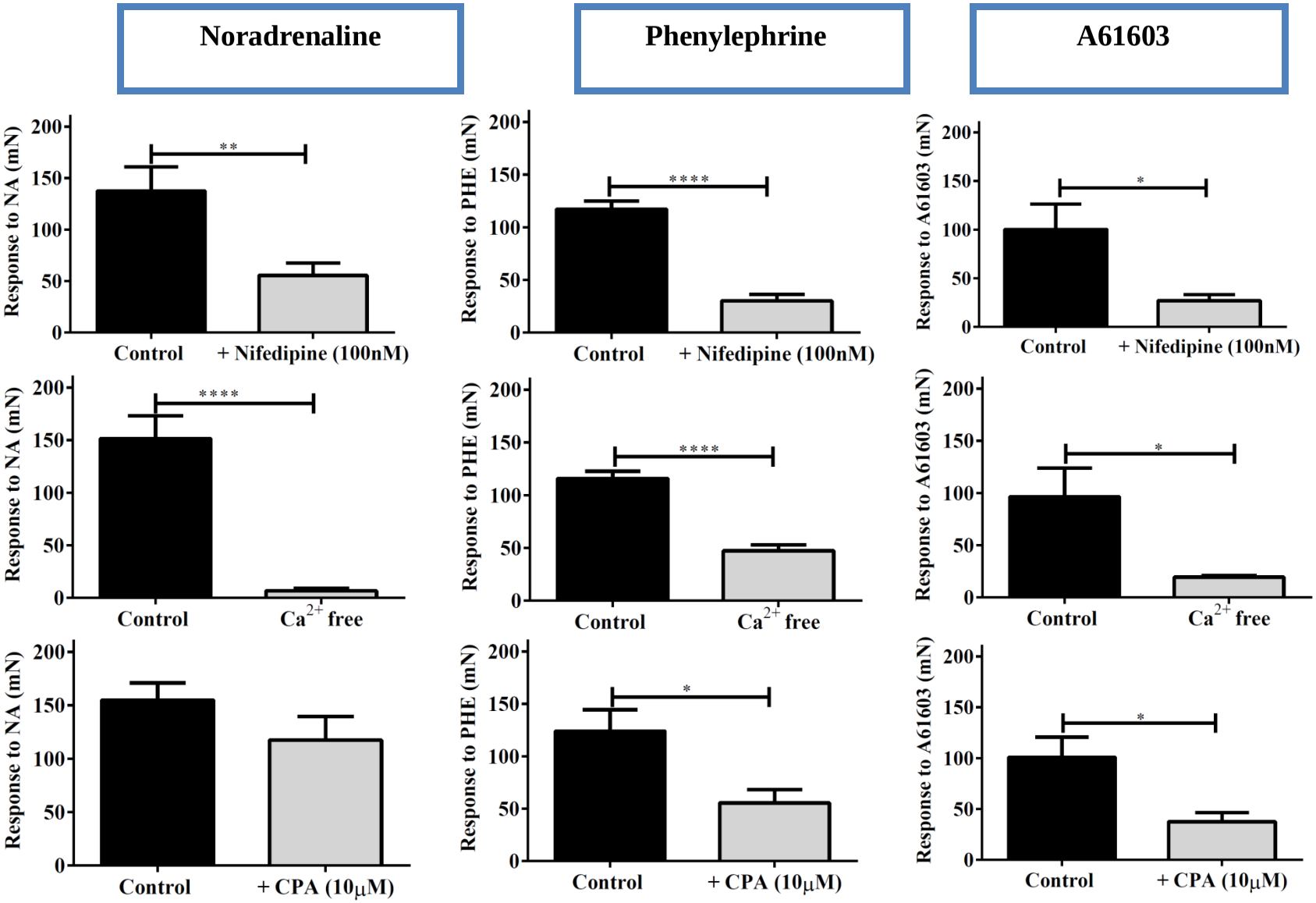


Figure 5.10 Mean (±SEM) equivalent contractile responses to noradrenaline, phenylephrine and A61603 in the presence or absence of inhibitor blocking the L-type Ca^{2+} channel, Ca^{2+} influx from the extracellular space and sarcoplasmic reticulum Ca^{2+} release (proximal tissues with urothelium/lamina propria). n=6-8. *P<0.05, ** P<0.01, and ****p<0.0001 vs. control (Student's t-test). NA= noradrenaline, PHE = phenylephrine.

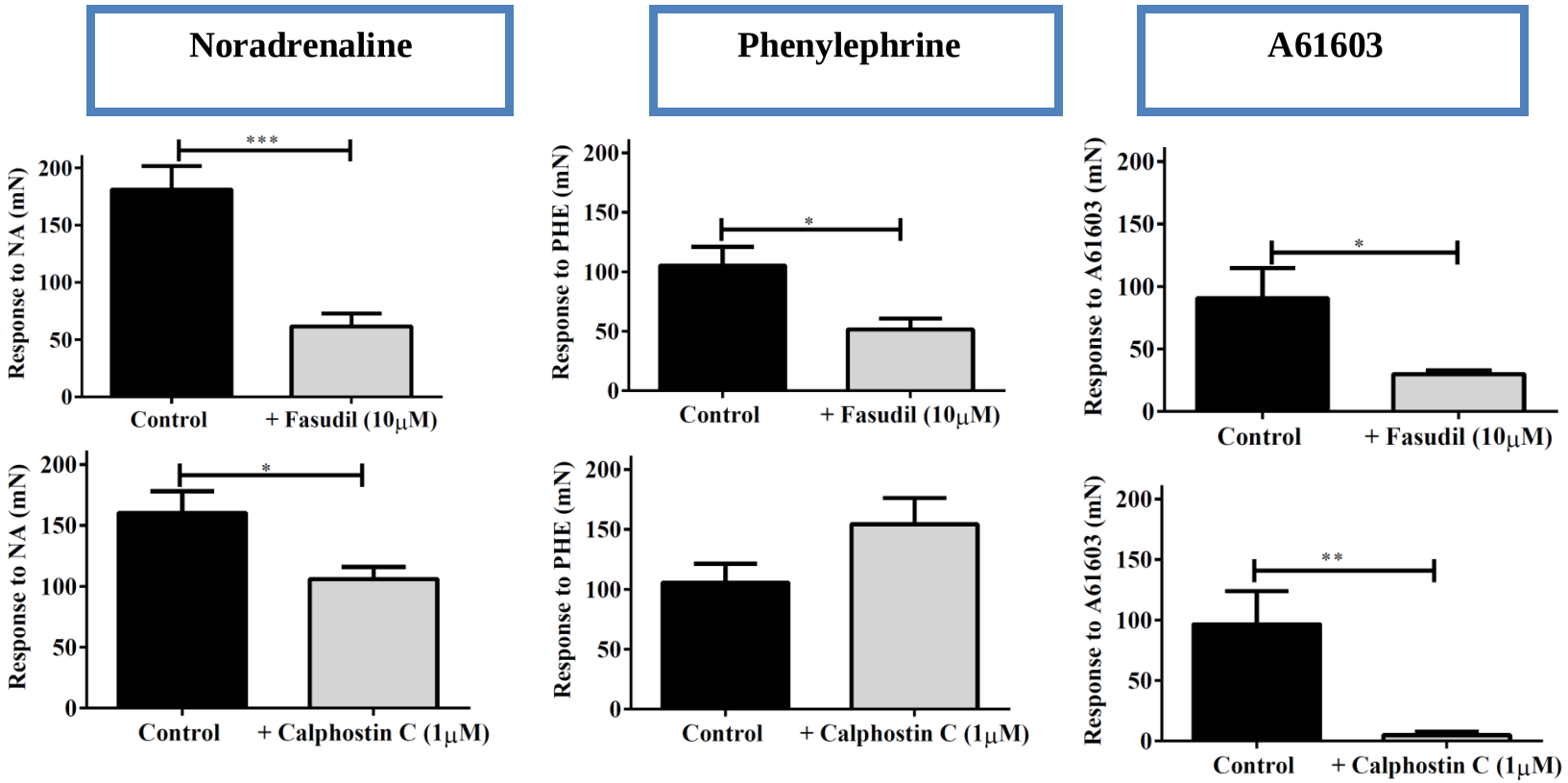


Figure 5.11 Mean (±SEM) equivalent contractile responses to noradrenaline, phenylephrine and A61603 in the presence or absence of inhibitor blocking the Rho kinase and PKC (proximal tissues with urothelium/lamina propria). n=6-8. *P<0.05, ** P<0.01, and ***p<0.001 vs. control (Student's t-test). NA= noradrenaline, PHE = phenylephrine.

Signalling pathways involved in producing half maximal responses

Incubation with inhibitors reduced half maximal responses to noradrenaline (Table 5.8A; Figure 5.12, Figure 5.13). Exclusion of Ca^{2+} from the bathing solution had the greatest effect on noradrenaline-induced contractions ($p<0.0001$). Incubation with nifedipine, CPA, fasudil and calphostin C also had inhibitory effects on the half maximal responses to noradrenaline, while calphostin C exerted no statistically significant effect.

Also, half maximal contractile responses to phenylephrine were significantly reduced by nifedipine, Ca^{2+} free/EDTA, CPA and fasudil (Table 5.8B; Figure 5.12, Figure 5.13). However, incubation with calphostin C (1μM) did not have any significant effect on half maximal responses to phenylephrine.

Lastly, half maximal contractile responses to A61603 were significantly reduced by all inhibitors (Table 5.8C; Figure 5.12, Figure 5.13). Similar to the effect on equivalent responses, incubation with calphostin C had the greatest inhibitory effect ($p<0.01$) on A61603.

(A)Noradrenaline	+ Nifedipine		Ca^{2+} free/EDTA		+ CPA		+ Fasudil		+ Calphostin C	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Response (mN)	100.4±18.8	**36.7±10.1****	112.0±17.1	**1.1±1.1 ******	101.3±17.3	78.6±19.2	120.5±17.8	**24.4±9.0*****	108.5±18.8	67.8±16.3
Percentage reduction in Max (%)	63.5±10.1		**99.0±1.0+++**		22.4±18.9		79.8±7.5		37.5±15.0	
n	8		7		7		8		6	

(B) Phenylephrine	+ Nifedipine		Ca^{2+} free/EDTA		+ CPA		+ Fasudil		+ Calphostin C	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Response (mN)	80.3±7.8	**7.0±2.8******	81.4±7.4	**12.3.±6.5 ********	86.1±15.5	**38.8±9.8***	67.8±14.4	**20.0±4.9***	68.3±14.5	105.1±21.3
Percentage reduction in Max (%)	91.2±3.5		84.9±7.9		55.0±11.4		70.4±7.2		+53.9±31.2	
n	8		8		8		6		6	

(C) A61603	+ Nifedipine		Ca^{2+} free/EDTA		+ CPA		+ Fasudil		+ Calphostin C	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Response (mN)	270.5±70.5	**73.2±16.9***	260.0±74.7	**52.6.±4.3***	272.0±53.8	**101.2±24.1***	244.7±64.9	**81.1±7.4***	260.4±74.6	**13.5±8.2****
Percentage reduction in Max (%)	72.9±6.2		79.8±1.6		62.8±8.9		66.9±3.0		**94.8±3.1++**	
n	6		6		8		7		6	

Table 5.8 Mean (±SEM) half maximal contractile responses and percentage reduction in the response of urethral tissues to noradrenaline, phenylephrine and A61603 in the presence or absence of inhibitor (proximal tissues with urothelium/lamina propria). *P<0.05, **p<0.01, ***p<0.001, and **** P<0.0001 vs. control (Student's t-test). ++p<0.01 and +++p<0.001 (One-way ANOVA with Bonferroni post-hoc test). Positive value= response of test tissue was greater than control tissue.

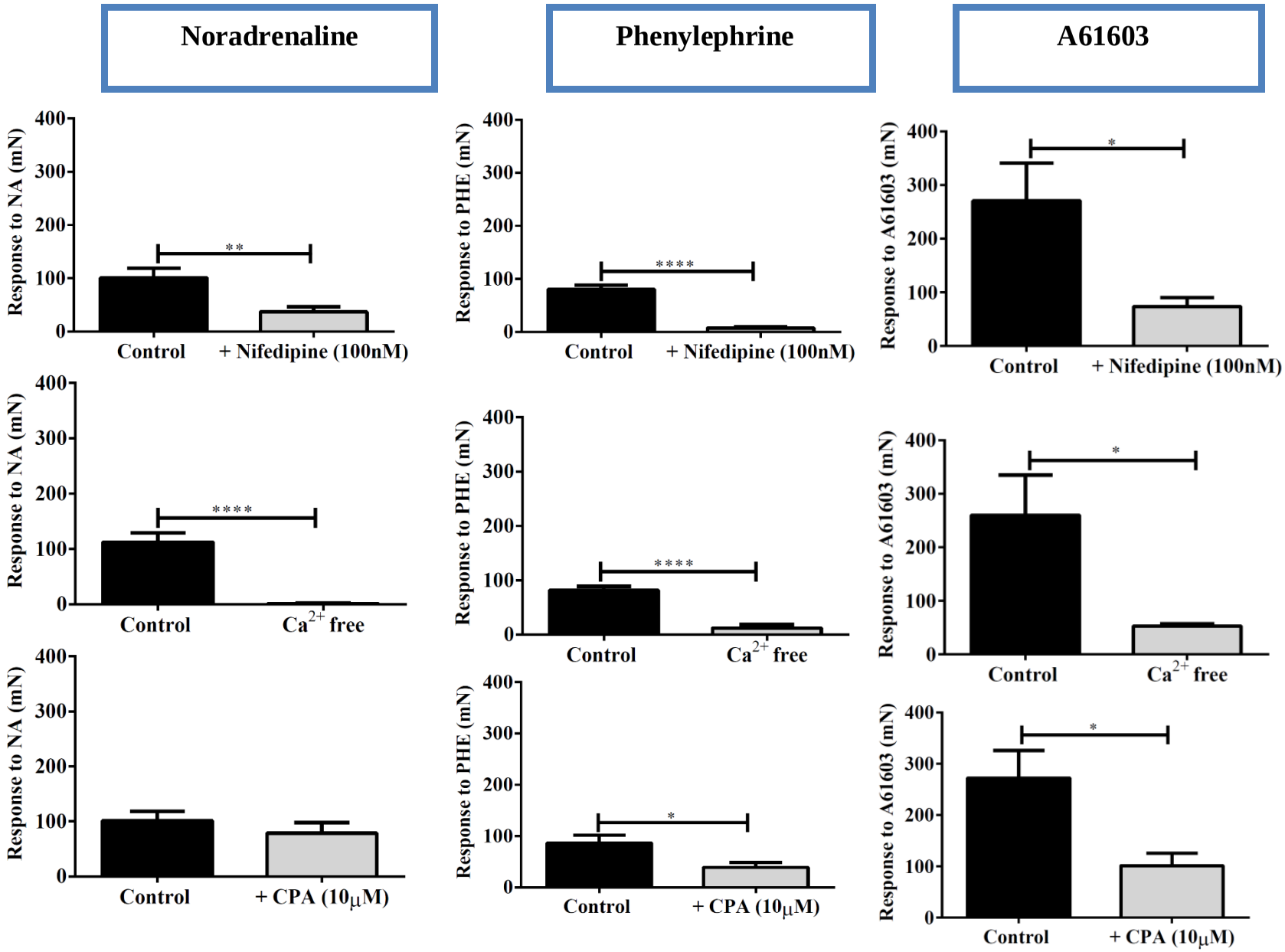


Figure 5.12 Mean (±SEM) half maximal contractile responses to noradrenaline, phenylephrine and A61603 in the presence or absence of inhibitor blocking the L-type Ca^{2+} channel, Ca^{2+} influx from the extracellular space and sarcoplasmic reticulum Ca^{2+} release (proximal tissues with urothelium/lamina propria). n=6-8. *P<0.05, ** P<0.01, and ****p<0.0001 vs. control (Student's t-test). NA= noradrenaline, PHE = phenylephrine.

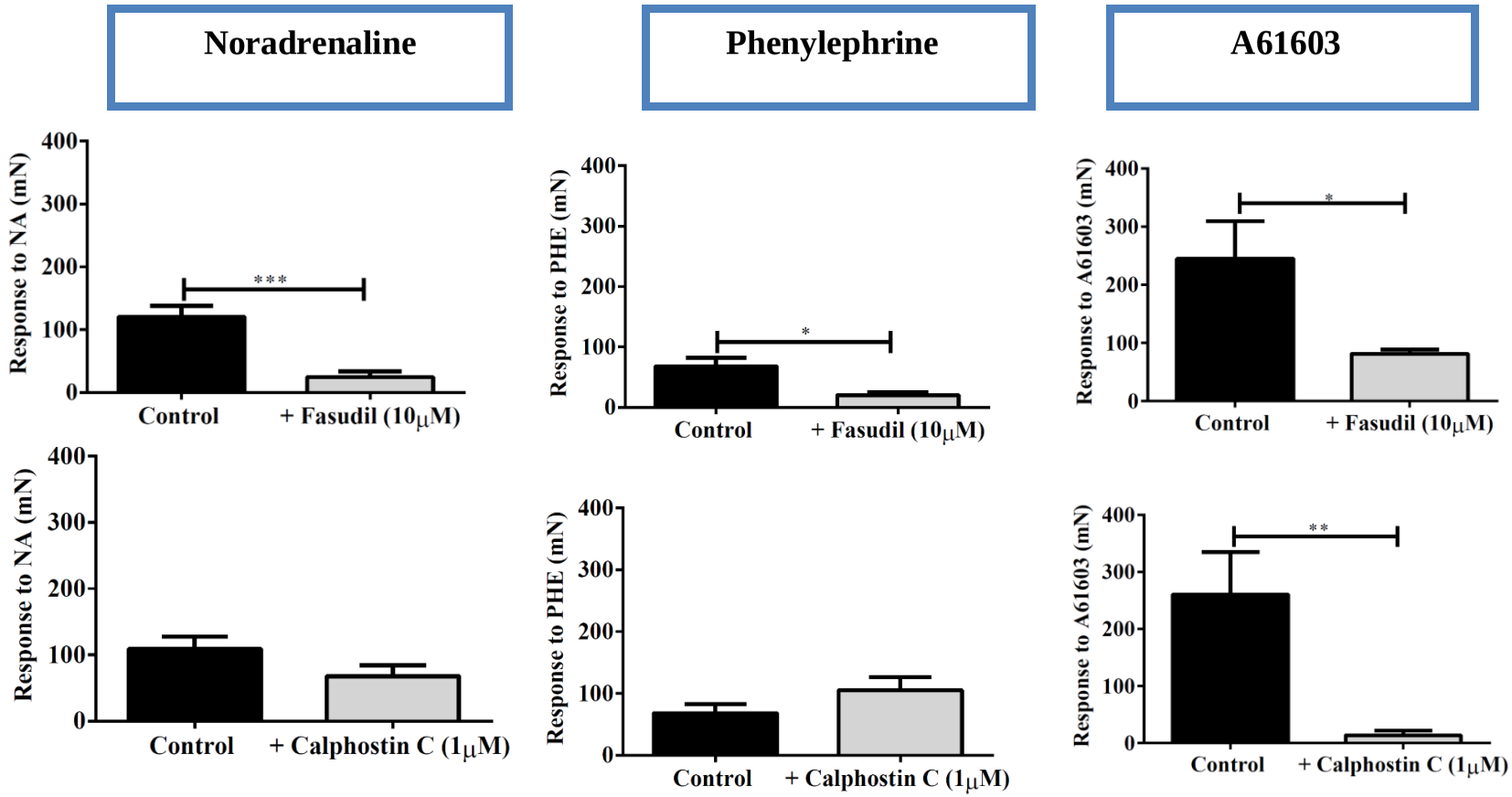


Figure 5.13 Mean (±SEM) half maximal contractile responses to noradrenaline, phenylephrine and A61603 in the presence or absence of inhibitor blocking the Rho kinase and PKC (proximal tissues with urothelium/lamina propria). n=6-8. *P<0.05, ** P<0.01, and ***p<0.001 vs. control (Student's t-test). NA= noradrenaline, PHE = phenylephrine.

Signalling pathways involved in producing three-quarter of maximal responses

Three-quarter maximal response of the tissues to noradrenaline and phenylephrine in the presence or absence of inhibitors was similar to that for equivalent responses (Table 5.9A-B; Figure 5.14, Figure 5.15). Also, all inhibitors reduced the three-quarter maximal contractile responses to A61603 (Table 5.9C; Figure 5.14, Figure 5.15). The inhibitory effect on A61603 induced contraction was greatest with calphostin C ($p<0.01$).

(A)Noradrenaline	+ Nifedipine		Ca^{2+} free/EDTA		+ CPA		+ Fasudil		+ Calphostin C	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Response (mN)	137.5±23.4	**55.5±12.1****	151.2±22.0	**6.8.±2.4 ******	154.8±16.0	117.4±22.1	181.0±20.7	**61.6±11.3*****	160.2±17.8	**105.9±10.0***
Percentage reduction in Max (%)	53.4±7.3		**95.5±1.6++++**		24.1±14.3		66.0±6.3		33.9±6.2	
n	8		7		7		8		6	

(B) Phenylephrine	+ Nifedipine		Ca^{2+} free/EDTA		+ CPA		+ Fasudil		+ Calphostin C	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Response (mN)	117.0±8.0	**30.1±6.2 ******	115.8±7.0	**47.3.±5.6 ******	123.9±20.5	**55.5±12.7 ***	105.0±15.9	**51.5±9.3***	105.4±16.0	154.2±21.9
Percentage reduction in Max (%)	74.3±5.3		59.1±4.9		55.2±10.2		51.0±8.8		+46.4±20.8	
n	8		8		8		6		6	

(C) A61603	+ Nifedipine		Ca^{2+} free/EDTA		+ CPA		+ Fasudil		+ Calphostin C	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Response (mN)	516.9±79.6	202.0±40.6*	476.6±93.5	171.0.±26.6 *	459.5±66.6	161.7±27.2 **	475.4±79.0	235.1±25.3*	507.2±85.6	35.7±23.0***
Percentage reduction in Max (%)	60.9±7.9		64.1±5.6		64.8±5.9		50.5±5.3		**93.0±4.5+++**	
n	6		6		8		7		6	

Table 5.9 Mean (±SEM) three-quarter maximal contractile responses of urethral tissues to noradrenaline, phenylephrine and A61603 in the presence or absence of inhibitor (proximal tissues with urothelium/lamina propria). *P<0.05, ** P<0.01, ***p<0.001 and ****p<0.0001 vs. control (Student's t-test). +++p<0.001 and ++++p<0.001 (One-way ANOVA with Bonferroni post-hoc test). Positive value= response of test tissue was greater than control tissue.

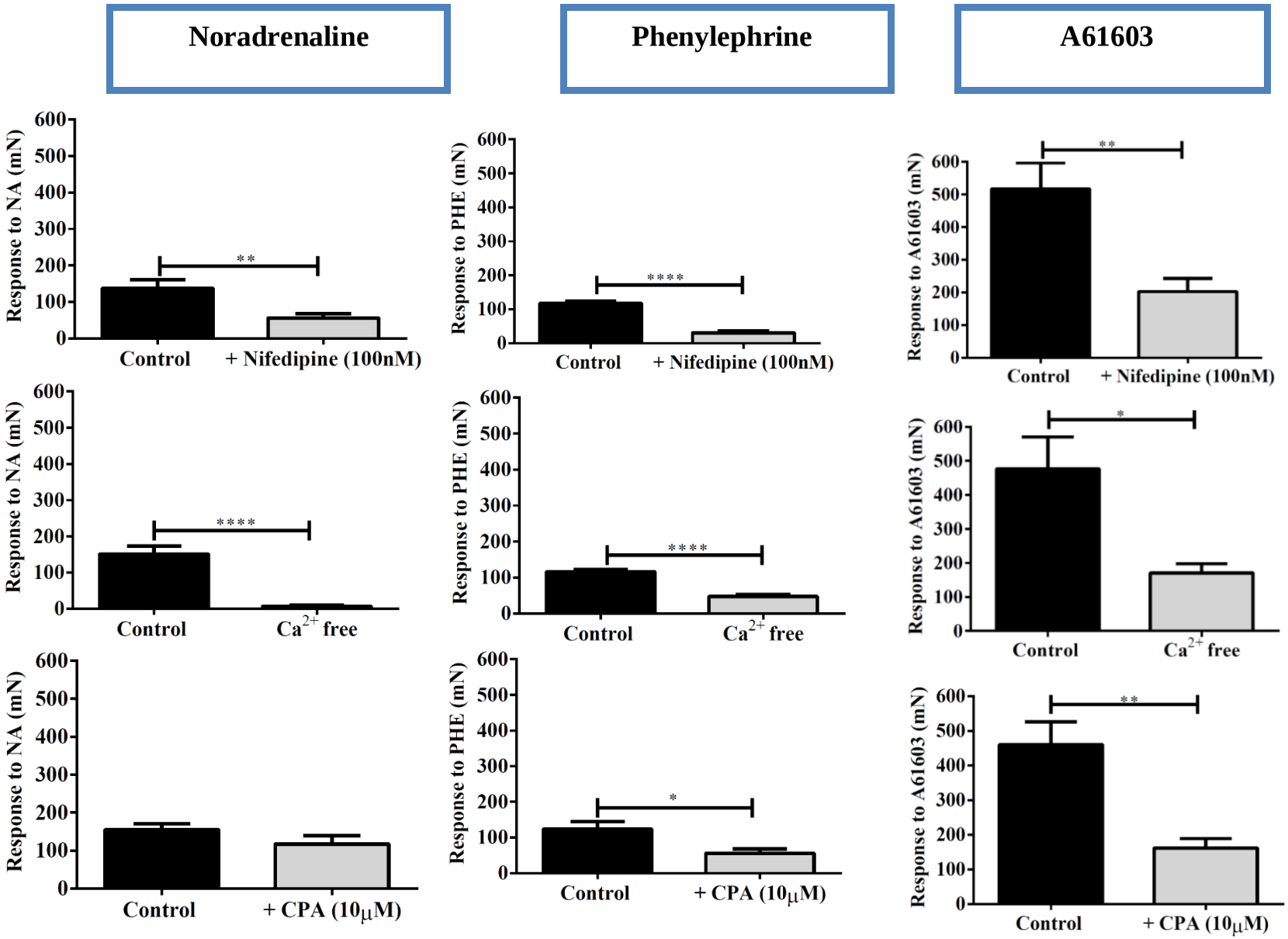


Figure 5.14 Mean (±SEM) three-quarter maximal contractile responses to noradrenaline, phenylephrine and A61603 in the presence or absence of inhibitor blocking the L-type Ca^{2+} channel, Ca^{2+} influx from the extracellular space and sarcoplasmic reticulum Ca^{2+} release (proximal tissues with urothelium/lamina propria). n=6-8. *P<0.05, ** P<0.01, and ****p<0.0001 vs. control (Student's t-test). NA= noradrenaline, PHE = phenylephrine.

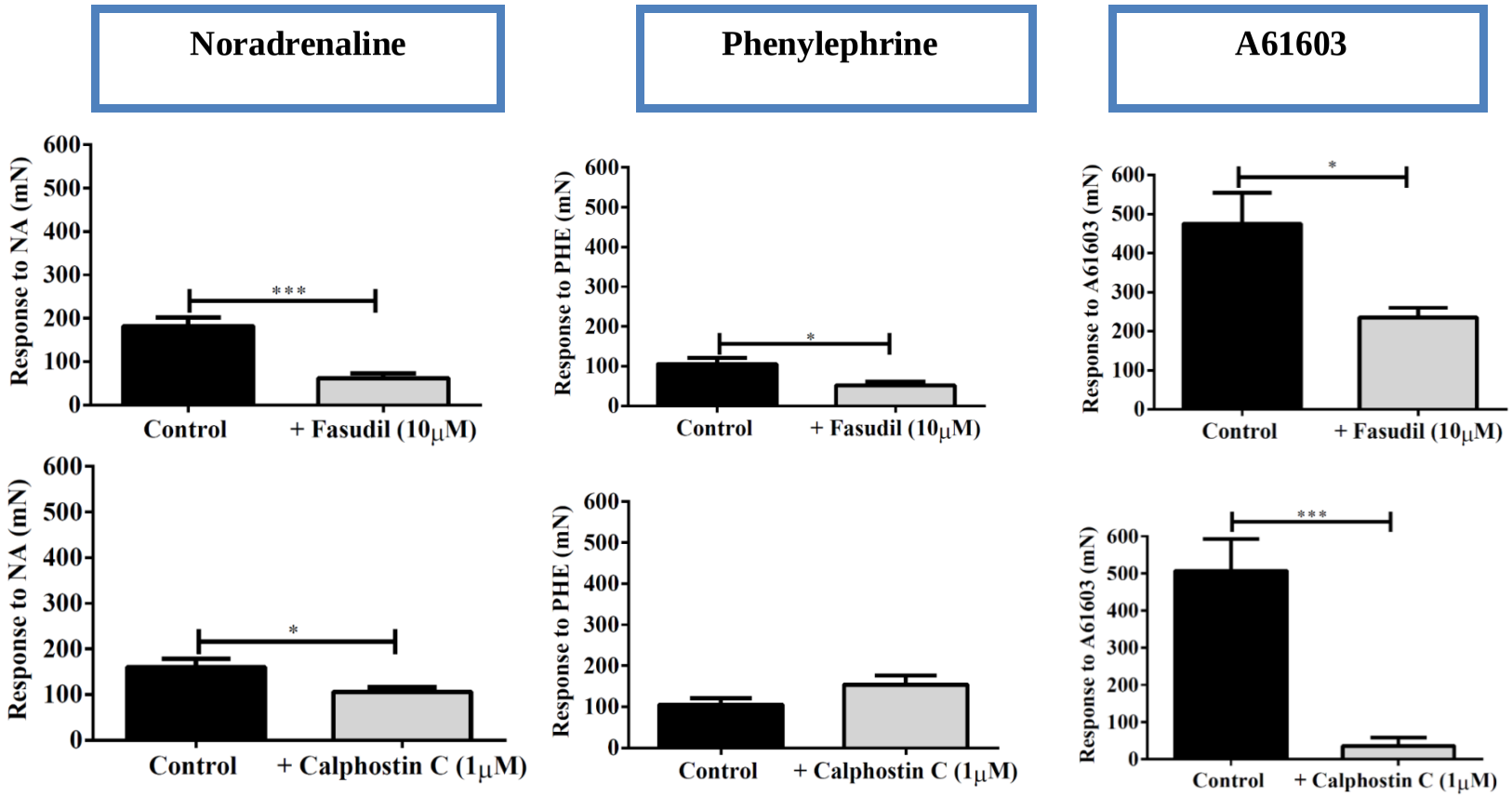


Figure 5.15 Mean (±SEM) three-quarter maximal contractile responses to noradrenaline, phenylephrine and A61603 in the presence or absence of inhibitor blocking the Rho kinase and protein kinase C (proximal tissues with urothelium/lamina propria). n=6-8. *P<0.05, and ***p<0.001 vs. control (Student's t-test). NA= noradrenaline, PHE = phenylephrine.

5.7 DISCUSSION

Davies et al., (2000) showed that the potency of fasudil and Y27632 are similar, and they have similar ability to inhibit Rho kinase, which implies that these inhibitors can be used interchangeably and either will produce a similar and reproducible result. In this report, the two Rho kinase inhibitors induced similar and reproducible inhibition of Rho kinase. Thus, fasudil (10μM) was used (except where stated otherwise) for Rho kinase inhibition in these studies because its inhibitory effect on Rho kinase is well reported in the literature.

Which signalling pathway mediates maintenance of urethral intrinsic basal tone?

The maintenance of urethral luminal pressure depends on basal urethral smooth muscle tone (Malmqvist et al., 2004; Werkstrom et al., 1995). It is known that urethral muscle generates a high myogenic tone in the pig (Bridgewater et al., 1993; Malmqvist et al., 2004), sheep (Thornbury et al., 1992), rat (McKeag et al., 2001) and human (Brading et al., 1999). However, to our knowledge the underlying signalling pathway is not known for the porcine model of the urethra. Voltage-dependent Ca^{2+} currents have been demonstrated in urethral myocytes of human and animals (Bradley et al., 2004; Hollywood et al., 2003a). Also, urethral/trigone tissues express RhoA/Rho kinase signalling components (Teixeira et al., 2007) and PKC (Roosen et al., 2008). Thus, we hypothesised that basal urethral tone might arise from a sustained elevation in intracellular Ca^{2+} and Ca^{2+} sensitization.

The data presented in this study revealed the development of basal tone in all tissue strips during the equilibration period. Exclusion of extracellular Ca^{2+} and incubation with EDTA (3mM) significantly decreased the basal urethral tone, confirming a previous report by Malmqvist et al., (2004). Blockade of L-type Ca^{2+} channels with nifedipine reduced the basal tone significantly and confirmed their essential role in generating a spontaneous basal tone. Several reports have shown that the urethral myogenic tone is critically dependent on the influx of Ca^{2+} across the cell membrane. The removal of external Ca^{2+} or inhibition of L-type Ca^{2+} channels reduces tone significantly in rat and human urethra in vitro (Brading, 1999; Shafei et al., 2003). In contrast, Mawby et al., (1991) showed that L-type Ca^{2+}-channels do not contribute to urethral luminal pressure in the cat suggesting possible species differences.

The inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase (exhaustion of sarcoplasmic reticulum Ca^{2+} store) with CPA did not have any significant effect on basal urethral tone suggesting that basal tone is dependent only on extracellular Ca^{2+} influx and not intracellular sarcoplasmic reticulum Ca^{2+} release in the pig urethra.

Rho kinase is essential in the maintenance of basal tone of hollow organs in rat (Patel & Rattan, 2006; Rattan et al., 2006) and human (Rattan & Singh, 2012). In the current study Y27632 and fasudil, which are selective inhibitors of Rho kinase (Uehata et al., 1997; He et al., 2008b), abolished the basal urethral tone and induced relaxation below basal tension. Both fasudil and Y27632 produced a similar result, which confirm the contribution of Rho kinase to basal urethral tone (Malmqvist et al., 2004). This study supports an earlier report showing that Toxin B, which specifically glucosylates Rho and disrupts its effector interaction, completely abolished pig urethral tone, strongly arguing that monomeric Rho guanosine triphosphatases in the Rho family have an essential role in the generation of urethral tone (Malmqvist et al., 2004).

Data from previous studies in smooth muscle such as the urethra (Teixeira et al., 2007), and vascular smooth muscle (Alvarez et al., 2010; Clelland et al., 2011: Ratz and Miner 2009; Ratz et al., 2009), have suggested constitutive Rho kinase activity which regulate basal phosphorylation of MYPT1. Thus, constitutively active Rho kinase may maintain myosin light chain phosphatase activity at a low level, resulting in a basal level of phosphorylated myosin light chain (as seen in the rabbit artery by Alvarez et al., (2010)), and contributing to the basal tone.

The possibility of PKC contributing to basal urethral tone was also addressed in the current study. Both Rho kinase and PKC signalling are both associated with Ca^{2+} sensitisation (Kitazawa et al., 2000; Kitazawa et al., 1999). Attenuation of PKC-induced contraction of smooth muscle by an inhibitor of Rho kinase II (though in the IAS) suggests that the PKC pathway involves Rho kinase activation (Patel & Rattan, 2007). In agreement with this, Baek et al., (2009) showed that PKC-mediated contractions of vascular smooth muscle contractions are mediated primarily via Rho kinase activation (Baek et al., 2009; Kandabashi et al., 2003). Thus, it was hypothesised that PKC may contribute to basal urethral tone. Surprisingly, inhibition of PKC with calphostin C (1μM) had no significant effect on the basal urethral

tone. This supports studies in the bladder, where animal studies suggested that PKC plays a minimal role in contractility (Schneider et al., 2004a). In addition, earlier studies testing the effects of selective inhibition of PKC in tissues of the gastrointestinal tract with basal tone, such as the lower oesophagal sphincter and IAS, suggested that PKC contributes little or nothing to the basal tone (Chakder et al., 2001; Kim et al., 2004). Thus, PKC may play a minor role in mediating basal tone in the hollow organs such as the urethra.

The urethra expresses cyclooxygenase enzyme, as shown in the dog (Ponglowhapan et al., 2009). Moreover, the dog urethra expresses prostaglandin receptors (Ponglowhapan et al., 2010), and prostaglandin-mediated relaxations and contractions have been shown in the rat (Kurihara et al., 2016) and human (Kedia et al., 2013) urethra. The human proximal urethral smooth muscle contracts to prostaglandin $F_{2\alpha}$, and relaxes to prostaglandin E_1 and prostaglandin E_2 (Andersson, et al. 1977). Thus, we tested the effect of inhibiting prostaglandins synthesis on basal urethral tone. Inhibition of cyclooxygenase reduced the basal urethral tone significantly, suggesting the contribution of prostaglandins in the maintenance of tone. Prostaglandins may mediate the basal tone via Ca^{2+} influx through the L-Type Ca^{2+} channels and activation of Ca^{2+} sensitization, since Rho kinase has been shown to mediate prostaglandin $F_{2\alpha}$ -induced contraction of the rabbit aorta (Ito et al., 2003). Thus, localised release of prostaglandins from urethral tissues may contribute to basal tone via L-type Ca^{2+} influx and Rho kinase. The PKC or intracellular Ca^{2+} released from the sarcoplasmic reticulum is not obligatory for generating a basal urethral tone in the pig.

What are the intracellular pathways associated with α_1-adrenoceptor-mediated responses?

The α-AR subtypes mediate an increase in cytosolic Ca^{2+}, and Keffel et al., (2000) showed in CHO cells that the maximum elevation of intracellular Ca^{2+} by α_1-AR subtypes occurred with the rank order $\alpha_{1A} > \alpha_{1D} > \alpha_{1B}$. Furthermore, trigone myocytes respond to phenylephrine by increasing force and intracellular Ca^{2+} (Roosen et al., 2008). However, the specific Ca^{2+}-channel and other intracellular pathways contributing to α_1-AR-mediated responses in the urethra remain to be elucidated.

In the previous chapter, we showed that A61603 induced the greatest contractile responses in the urethra and had the greatest potency compared to noradrenaline and phenylephrine. Moreover, the literature has shown that A61603 has greater specificity for α_{1A}-AR (Knepper et al., 1995). Thus, we intended to ascertain whether the receptor-coupled intracellular signalling pathways might explain the difference in potency.

L-type Ca^{2+} channels contributed only 60.1% (Figure 5.5) of the total extracellular Ca^{2+} to responses to noradrenaline, suggesting other means of Ca^{2+} increase are involved. T-type Ca^{2+} currents have been recorded in rabbit and human urethral myocytes, but these mediate spontaneous contractions (Bradley et al., 2004; Hollywood et al., 2003a). Although T-type Ca^{2+} channels activate at more negative potentials than L-type Ca^{2+} channels, they facilitate L-type Ca^{2+} channels opening (Sui et al., 2003). Thus, if this is the case in these experiments, then noradrenaline may have activated T-type Ca^{2+} channels, which may trigger further opening of the L-type Ca^{2+} channels. The reverse-mode Na^{+}-Ca^{2+} exchanger is another possible channel allowing Ca^{2+} influx from the extracellular environment (Bradley et al., 2006; Drumm et al., 2015). The function of this exchanger as a mediator of Ca^{2+} waves has been shown in ICC isolated from the rabbit urethra (Drumm et al., 2015); though whether this channel is important in the pig urethra is not clear.

In contrast, for A61603 and phenylephrine-induced contractions, the L-type Ca^{2+} channels mediate Ca^{2+} influx from the extracellular space. This is similar to the trigone where the influx of Ca^{2+} is via the L-type Ca^{2+} channels in response to α_{1A}-AR stimulation (Roosen et al., 2009).

The Rho kinase pathway has been shown to contribute to rat (Teixeira et al., 2007) and rabbit (Walsh et al., 2011) urethral contraction. From our experiments, we observed that the Rho kinase also contributes to agonist-mediated responses in the pig urethra and this was similar for all agonists. PKC significantly contributed to responses mediated by A61603 but not to phenylephrine-induced contraction. Walsh et al., (2011) also showed that PKC inhibition had no effect on phenylephrine-induced, KCl-induced or electrical field stimulation-induced contraction although in the rabbit. These findings together suggest that PKC contributes little to phenylephrine-induced contraction in the urethra. In the bladder, the role of the PKC on contractility appears to be species dependent with a role in guinea-pig tissues (Durlu-Kandilci & Brading, 2006) but not other species (An et al., 2002; Schneider et al., 2004b).

Thus responses to A61603 appear to involve both Rho kinase and PKC in the Ca^{2+}sensitization pathway. Earlier data, although not from urethral tissues, suggests that Rho kinase and PKC pathways lie in series, PKC being upstream of RhoA/Rho kinase (Sims et al., 2008; Wang et al., 2012). Activation of PKC will induce Ca^{2+} sensitization via CPI-17 and Rho kinase (a synergistic effect) (Wang et al., 2012). Thus may explain the greater potency and efficacy of A61603 compared to noradrenaline and phenylephrine and agonists depending solely on Rho kinase (e.g. phenylephrine) may induce smaller contractile responses.

Signalling pathways contributing to α_1-adrenoeptor-mediated responses in the urethral tissues; comparing equivalent, half maximal, three-quarter maximal and maximal responses

In the previous section, we showed that the contributions of the intracellular signalling pathway to contractile responses differ between α_1-AR agonists. However, this conclusion was based on comparison of the maximum contractile responses induced by the agonists. Given the differences in potency, there is the possibility the signalling pathways triggered by the agonists may differ depending on the magnitude of the response.

For maximal responses to noradrenaline, extracellular Ca^{2+} was the most significant contributor whilst intracellular sarcoplasmic reticulum released Ca^{2+} contributed less. Specifically, the L-type Ca^{2+} channels contributed (60.1%) to extracellular Ca^{2+} influx for this response. However, in contrast sarcoplasmic reticulum released Ca^{2+} and PKC contributed most to phenylephrine- and A61603-induced contractile responses respectively. Similarly, at half maximal responses induced by agonists, extracellular Ca^{2+} was the most significant contributor, while sarcoplasmic reticulum released Ca^{2+} contributed less to noradrenaline-mediated responses. Specifically, the L-type Ca^{2+} channels contributed (64.1%) to the extracellular Ca^{2+} influx. L-type Ca^{2+} and PKC contributed most to phenylephrine- and A61603-induced contractile responses respectively. At three-quarter maximal responses, extracellular Ca^{2+} was still the most significant contributor to noradrenaline-mediated responses. While L-type Ca^{2+} and PKC contributed most to phenylephrine- and A61603-induced responses respectively.

When looking at equivalent responses induced by the agonists, extracellular Ca^{2+}, L-type Ca^{2+} and PKC were still the most significant contributor to noradrenaline-, phenylephrine- and A61603-mediated responses respectively. Specifically, the L-type Ca^{2+} channels contributed (64.1%; Figure 5.10) to the extracellular Ca^{2+} influx for noradrenaline-mediated responses. Thus, for all responses at all concentrations, extracellular Ca^{2+} plays a significant contribution to agonist-mediated responses. Sarcoplasmic reticulum released Ca^{2+} seems to be more important for the smooth muscle spontaneous contractions as shown in the rabbit urethral circular smooth muscle (Hashitani et al., 2006).

This finding is similar to that seen for mAChR activation in the bladder, where the L-type Ca^{2+} channels and calcium sensitization (Durlu-Kandilci and Brading, 2006) were most important for receptor-mediated responses (Frazier et al., 2008; Schneider et al., 2004b). Ablation of L-type Ca^{2+} channels in the mouse resulted in a decrease in the protein expression of myosin light chain (Lu et al., 2015), and Martinsen et al., (2014) showed myosin light chain kinase controlled mRNA expression of L-type Ca^{2+} channels in vascular smooth muscle cells, which suggest a relationship between muscle contraction and L-type Ca^{2+} channels.

For noradrenaline at all responses, extracellular Ca^{2+} was the most significant contributor to contraction while the contribution of Rho kinase and PKC varies between different responses. Extracellular Ca^{2+} influx and Ca^{2+} sensitization mediate the noradrenaline-induced contraction at half maximal and maximal responses. This result supports an earlier report stating that agonist-induced contraction of rat ventricular cells and trigone of the guinea-pigs largely depends on Ca^{2+} entry from the extracellular area (Liu et al., 1994) and Ca^{2+} sensitization (Roosen et al., 2008) respectively. The contributing intracellular pathways to phenylephrine-induced contractile response also vary with the concentration. Extracellular Ca^{2+} was more important at half maximal response and three-quarter maximal and equivalent responses. However, for maximal responses to phenylephrine, sarcoplasmic reticulum Ca^{2+} release was the most important factor. The change from extracellular Ca^{2+} influx to sarcoplasmic reticulum Ca^{2+} release suggests Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (Collier et al., 2000). Ca^{2+}-induced Ca^{2+} release is a function of the net flux of Ca^{2+} ions into the cytosol (Collier et al., 2000).

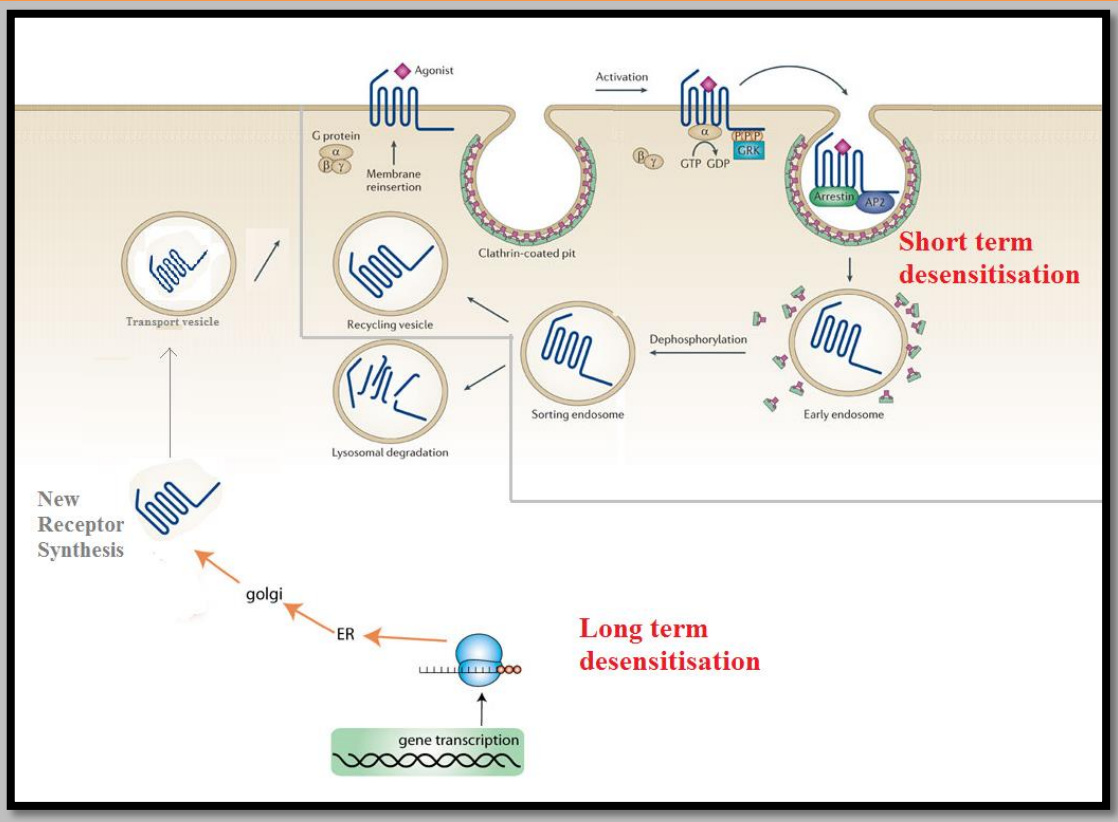
PKC did not contribute to phenylephrine-mediated responses. Additionally, PKC may have an inhibitory effect on phenylephrine-induced contractions; an inference made based on the potentiation of responses in the presence of PKC inhibitor, although this effect was not statistically significant. In contrast, A61603-induced contractions were significantly dependent on PKC at all concentrations analysed. Hypolite et al., (2015) showed in the bladder that low levels of PKC stimulation could inhibit spontaneous contractions and lower basal resting tone, while high levels of PKC activity contributed to increasing and maintenance of total force. In the present study phenylephrine may induce low PKC activation which could mediate inhibition of contractile responses or have no significant effect at equivalent, half maximal, three-quarter maximal and maximal responses, whereas A61603 and noradrenaline may induce high PKC activation which mediates contractions. A61603 may also induce greater activity of PKC than noradrenaline, which may explain PKC being the greatest contributor to A61603-mediated responses compared to noradrenaline. However, it is still unknown whether different PKC isoforms mediate the effect of the different agonists at various responses.

Thus, responses of the urethral to agonists acting via the α_1-AR are determined by the relative intracellular signalling pathway activated. The intracellular signalling pathway contributing most to responses was different for phenylephrine at maximal responses, but did not differ for A61603 and noradrenaline at any response or concentration. PKC contributed most to A61603-mediated responses, while Ca^{2+} contributed most to noradrenaline and phenylephrine-mediated responses respectively.

Conclusion

- The basal tone of porcine urethral tissues is dependent on L-type Ca^{2+} channel influx, prostaglandins and Rho kinase activity. However, intracellular Ca^{2+} release from the sarcoplasmic reticulum and PKC does not contribute to basal tone.
- For noradrenaline, all contractile responses depend mainly on extracellular Ca^{2+} influx. For maximal responses to phenylephrine, intracellular Ca^{2+} stores contribute most to contractions.
- In contrast, contractile responses to A61603 depended mainly on Ca^{2+} -sensitization; PKC.

CHAPTER 6



Modified from Ramachandran et al., 2012.

6 DESENSITISATION AND MAINTENANCE OF TONE IN THE URETHRA

Alpha$_{1A}$-ARs are a potential drug target for increasing urethral luminal pressure in stress urinary incontinent patients (Noel et al., 2010; Segev et al., 2015). Fortunately, the α_{1A}-ARs appear to be less desensitised, internalised and downregulated than α_{1B}-ARs and α_{1D}-ARs (Cabrera-Wrooman et al., 2010; Stanasila et al., 2008). Several drugs selective for the α_1-ARs have been developed. However, most drugs investigated have never made it to clinical applications. Particularly, drugs with selectivity for the α_{1A}-subtype have been developed preclinically, including NS-49 (Obika et al., 1995), A-61603 (Knepper et al., 1995) and Ro 115–1240 (Blue et al., 2004). The latter compound was successfully tested in a proof-of-concept clinical study in stress incontinence patients (Musselman et al., 2004), but its clinical development was discontinued due to undisclosed reasons. One of the explanations for this set back may be desensitisation of the α_{1A}-ARs.

Since any future treatment to increase the urethral luminal pressure using α_1-AR agonists would involve the long-term usage of an agonist, this may mediate chronic activation and desensitisation of these receptors and thus, could worsen the symptoms of stress urinary incontinence.

6.1 ALPHA$_1$-ADRENOCEPTORS DESENSITISATION

The α_1-ARs are known to be subject to phosphorylation and internalisation on exposure to noradrenaline, or phenylephrine in Rat-1 fibroblasts cells (Vazquez-Prado et al., 2000). The α_{1A}-AR subtype has been observed in lipid rafts under basal conditions (Lei et al., 2009; Morris et al., 2008) and it undergoes constitutive and phenylephrine-mediated internalisation via clathrin-coated vesicles (Morris et al., 2004; Pediani et al., 2005; Price et al., 2002). The α_{1A}-AR exits from these rafts after stimulation with phenylephrine, therefore allowing endocytosis (Morris et al., 2008). The α_{1A}-AR stimulation causes the exit of the receptors from lipid rafts within 3-10 minutes in Rat-1 fibroblasts cells. However, G proteins remain in this raft suggesting that receptor signalling and desensitisation of α_{1A}-ARs occurs in the raft (Morris et al., 2008).

Human α_{1A}-AR-mediated inositol phosphate signalling is acutely desensitised in response to both agonist and phorbol 12-myristate 13-acetate (PKC activator) exposure in Rat-1 fibroblasts cells (Price et al., 2002). Concurrent with desensitisation, in response to both noradrenaline and phorbol 12-myristate 13-acetate, kinases mediate phosphorylation of the α_{1A}-ARs in the cells. Akinaga et al., (2013) observed using HEK-293 cells a GPCR-dependent α_{1A}-AR phosphorylation, desensitisation and internationalisation at least after 5 minutes of stimulation. Phosphorylation of α_{1A}-ARs exposed to noradrenaline depends on PKC activity and is not followed by desensitisation. Moreover, α_{1A}-ARs exposed to noradrenaline undergo delayed internalisation (~35% after 60 minutes of stimulation) (Akinaga et al., 2013). Oxymetazoline (α_1-AR agonist) also desensitise the α_{1A}-ARs in the rat tail artery and vas deferens, but this does not occur with noradrenaline or phenylephrine. This suggests that agonists with functional selectivity for α_{1A}-ARs lead to significant receptor desensitisation and internalisation.

Alpha$_{1A}$-ARs are phosphorylated and desensitised to a lesser extent on exposure to noradrenaline, adrenaline, or phenylephrine compared to the α_{1B} and α_{1D} subtypes (Cabrera-Wrooman et al., 2010; Vazquez-Prado et al., 2000). For example, α_{1B}-ARs are phosphorylated to a greater extent in response to either noradrenaline or tetradecanoyl phorbol acetate (PKC activation) than α_{1A}-ARs (Cabrera-Wrooman et al., 2010; Vazquez-Prado et al., 2000). Also, recombinant α_{1A}-ARs expressed in the Rat-1 fibroblasts are much more resistant to desensitisation induced by noradrenaline than are α_{1B} and α_{1D} subtypes (Cabrera-Wrooman et al., 2010; Vazquez-Prado et al., 2000).

Alpha$_{1A}$-ARs are also internalised less on exposure to noradrenaline or phenylephrine than α_{1B} and α_{1D} subtypes (Cabrera-Wrooman et al., 2010; Chalothorn et al., 2002; Stanasila et al., 2008; Wang et al., 2007). Phenylephrine stimulation promotes a more rapid internalisation of α_{1B}-ARs than α_{1A}-ARs in HEK-293 cells (Perez-Aso et al., 2013; Wang et al., 2007). Activation of α_{1A}-AR elicits rapid PKC-dependent and independent phosphorylation and internalisation associated with arrestin. However, α_{1B}-AR activation elicits PKC-dependent phosphorylation and internalisation. On the other hand, agonist activation of α_{1D}-AR elicits rapid, transient PKC-independent phosphorylation. Thus Perez-Aso and colleagues, (2013) result suggested that the three α_1-AR subtypes present different patterns of receptor phosphorylation and internalisation. Whole cell [^{3}H]-prazosin binding assays showed that

α_{1A}-AR functional receptors were detected both on the cell surface and in the cytoplasm; α_{1B}-ARs, however, were detected predominantly on the cell surface, while α_{1D}-ARs were detected mainly in intracellular compartments (Wang et al., 2007).

Thus, as far as receptor phosphorylation, desensitisation, internalisation and signalling are concerned, α_{1A}-ARs are less affected than the α_{1B} and α_{1D} subtypes (Cabrera-Wrooman et al., 2010; Chalothorn et al., 2002; Stanasila et al., 2008; Wang et al., 2007). Significant reductions in endocytosis recorded for α_{1A}-AR in HEK-293 cells has been linked to its poor interaction with arrestins as well as with AP50 subunit of the AP2 clathrin adaptor complex (Stanasila et al., 2008).

In addition, Segura et al., (2013) observed that constitutive as well as phenylephrine-induced trafficking of α_{1A}-ARs and α_{1B}-ARs maintain two different endosomal pools of receptors: one in proximity to the plasma membrane and the other farther into the cytosol of HEK-293 cells. The location of the receptors determines their signalling pathway. For example α_{1A}-ARs signal via Ca^{2+} pathways when located on the plasma membrane but, via arrestin when translocated to deeper endosomes. However, α_{1B}-ARs signal through Ca^{2+} pathways only when located in the membrane and the signals disappear after endocytosis (Segura et al., 2013).

Many lines of evidence suggest that activation of α_1-ARs also induces phosphorylation of MAPKs and extracellular signal-regulated kinase (Keffel et al., 2000; Wright et al., 2008). Moreover, trafficking, docking, and fusion with endosomes were made possible by the Rab (Rho guanosine triphosphatases), which characterises the endosome (Castillo-Badillo et al., 2015). In experiments performed using transfected Rat-1 fibroblasts that express α_{1B}-ARs in a stable fashion, selective epidermal growth factor-receptor kinase inhibition blocked the α_{1B}-AR phosphorylation induced by noradrenaline (Casas-González & García-Sáinz, 2006). This result suggested the epidermal growth factor receptors play a more general role in α_{1B}-AR phosphorylation.

In cultured neonatal rat cardiac myocytes and rats subjected to aortic banding, long-term activation of α_1-ARs increased the α_{1A}-AR, without desensitising α_1-AR-mediated inositol phosphate turnover or growth, while also decreasing α_{1B}-AR mRNA and protein levels

(Rokosh et al., 1996). One of the explanations for the differences in desensitisation of α_1-AR and β-ARs might reside in the expression and regulation of GRKs. For example, GRK3 has been found exclusively in myocytes, where it regulates α_1-ARs, (Vinge et al., 2001, 2007; Aguero et al., 2012). However, GRK2 and GRK5 desensitised β-ARs but not α_1-ARs (Vinge et al., 2001, 2007; Aguero et al., 2012). Thus, the difference in ARs trafficking evokes different signalling.

Desensitisation of the responses of the lower urinary tract smooth muscle

Desensitisation has been reported in bladder smooth muscle responses. For example, Cha et al., (2006) reported desensitisation of mAChRs of bladder smooth muscle strips isolated from mouse, where repeated pilocarpine injections revealed mAChR desensitisation to subsequent addition of drug. In contrast, stimulation of dog bladder strips with acetylcholine after prior incubation with bethanechol, induced no change in the responses, suggesting resistance of the mAChRs in the dog bladder to desensitisation (Buranakarl et al., 2001). The variation in result may be associated with species of animal used or degree of receptor stimulation, as shown by Cha et al., (2006). Six hours pre-treatment with a β_2-AR agonist significantly reduced subsequent relaxation to the agonist in the rat bladder (Michel, 2014). Moreover, agonist-induced desensitisation was prominent for the β_2-AR component of rat bladder relaxation but much weaker or even absent for the β_3-AR component (Michel, 2014). Thus, desensitisation of mAChR and β-AR-mediated responses occurs in the bladder.

Reports showing desensitisation of the urethral response are limited. However, Chess-Williams & Bagot, (2005) observed desensitisation of the α-AR-mediated responses in the porcine urethra. They compared the susceptibility of urethral and IAS smooth muscle and showed that the urethral responses to α_1-AR- agonists are more susceptible to desensitisation compared to the IAS. After incubation with A61603 or methoxamine and washout of the drug, subsequent concentration-response curves to noradrenaline were similar to controls in IAS. In contrast, urethral tissues were readily desensitised. Incubation of the urethral smooth muscle strips with EC_{50} concentrations of methoxamine or A61603 caused a desensitisation of subsequent responses to noradrenaline with maximum responses being depressed by 42-56%. Moreover, the α_1-AR-mediated desensitisation of the urethral tissues response is proportional to the concentration of agonist (unpublished result). Thus, we studied the effect of time of

incubation with α_1-AR agonist on the desensitisation and elucidated the signalling pathways modulating this mechanism.

Since little evidence of desensitisation is available for α_1-ARs in the urethral tissues, this study aimed to broaden understanding of desensitisation of α_1-AR-mediated responses in urethral tissues.

Specific objectives:

1. **To investigate the maintenance of tone in the porcine urethra following agonist exposure.**
    - Can urethral tone maintenance following a α_1-AR agonist be demonstrated in the pig urethra?
    - Is tone maintenance dependent on α-AR agonist selectivity?
    - Is the urothelium/LP involved in the maintenance of urethral tone?
    - Are Rho kinase and prostaglandins involved in the maintenance of urethral tone?
2. **To investigate the desensitisation of urethral responses following exposure to an α-AR agonist.**
    - Is desensitisation of α_1-AR-mediated responses dependent on the α-AR agonist?
    - Does the degree of desensitisation depend upon the length of agonist exposure?
3. **To investigate the intracellular mechanism involved in desensitisation.**
    - Do PKC, Rho kinase or Ca^{2+} contribute to desensitisation of α_1-AR-mediated responses, and to what extent?

6.2 MATERIALS AND METHODS

Maintenance of urethral tone following α_1-adrenoceptor stimulation: role of the urothelium/lamina propria/ Rho kinase and prostaglandins

Strips of female pig proximal urethra were set up in 8ml organ baths as described in chapter 2. Following set up and equilibration for 1 hour, tissues were contracted to a single concentration of noradrenaline (10μM), phenylephrine (10μM) or A61603 (0.03μM) and the contraction was recorded for 120 minutes. These concentrations were equi-efficient concentrations of the agonists (as determined in chapter 5).

To investigate the effect of the urothelium/ LP on desensitisation, tissues with or without urothelium/LP were set up in pairs and contracted to either phenylephrine (10μM) or A61603 (0.03μM) for 120 minutes. The role of Rho kinase and prostaglandins in tone maintenance was determined in tissue pairs. Test tissues were incubated with fasudil (10μM) or indomethacin (10μM) for 30 minutes and then contracted to a single concentration of phenylephrine (10μM) or A61603 (0.03μM) for 120 minutes.

Desensitisation of noradrenaline responses following α_1-adrenoceptor stimulation

Following set up and equilibration of tissue strips, initial cumulative concentration-response curves were constructed to noradrenaline. After the washout (1 hour, changing Krebs every 20 minutes) the tissues were incubated with an EC_{50} concentration of one of the agonists (noradrenaline, phenylephrine, A61603) for either 15, 30 or 120 minutes. The EC_{50} concentrations for the various α_1-AR agonists used were obtained from experiments carried out in chapter 4. At the end of the incubation period, the drugs were washed out until tensions returned to baseline (30 - 40 minutes). Tensions were recorded throughout the incubation period. Following washout, a second cumulative concentration-response curve to noradrenaline was produced (Figure 6.1). Responses were plotted as a percentage of the maximum contraction to noradrenaline achieved in that tissue during the first concentration-response curve. Control tissues without incubation with an α-AR agonist were used to allow correction for any time-dependent changes in maximum response or sensitivity (Figure 6.2).

In control experiments, there were similar maximum contractile responses to noradrenaline in first and second curves (Figure 6.2). However, the second response curves were significantly shifted to the right, thus, a correction factor was applied and used for normalising EC_{50} values to correct for time-dependent changes. Contribution of signalling pathways to urethral tone was determined in intact tissues.

Role of intracellular pathways in desensitisation

In separate experiments, urethral tissues were incubated throughout the experiments with an inhibitor of key molecules involved in intracellular pathways in the urethral smooth muscle (Figure 6.1). Fasudil (10μM), CPA (10μM), nifedipine (1μM), calphostin C (1μM) was used to block Rho kinase, intracellular release of Ca^{2+} from the sarcoplasmic reticulum, L-type Ca^{2+} channels Ca^{2+} influx or PKC respectively (Figure 6.3).

Following equilibration, tissues were contracted to cumulative concentrations of noradrenaline (Figure 6.1). After washout (30 minutes, changing Krebs every 10 minutes) the tissues were incubated with an EC_{50} concentration of one of the α_1-AR agonists (phenylephrine or A61603). Since similar desensitisation of α_1-AR-mediated responses was observed at 15 minutes, 30 minutes and 120 minutes (Figure 6.2), 15 minutes incubation was used in experiments reported in this section. At the end of the incubation period, the drugs were washed out four times and then every 10 minutes until tensions returned to baseline. Following the washout, a second cumulative concentration-response curve to noradrenaline was produced in all the tissues. Control tissues without inhibitor were used to allow for time-dependent changes. The α_1-AR-mediated responses were analysed and data expressed as described in chapter 2.

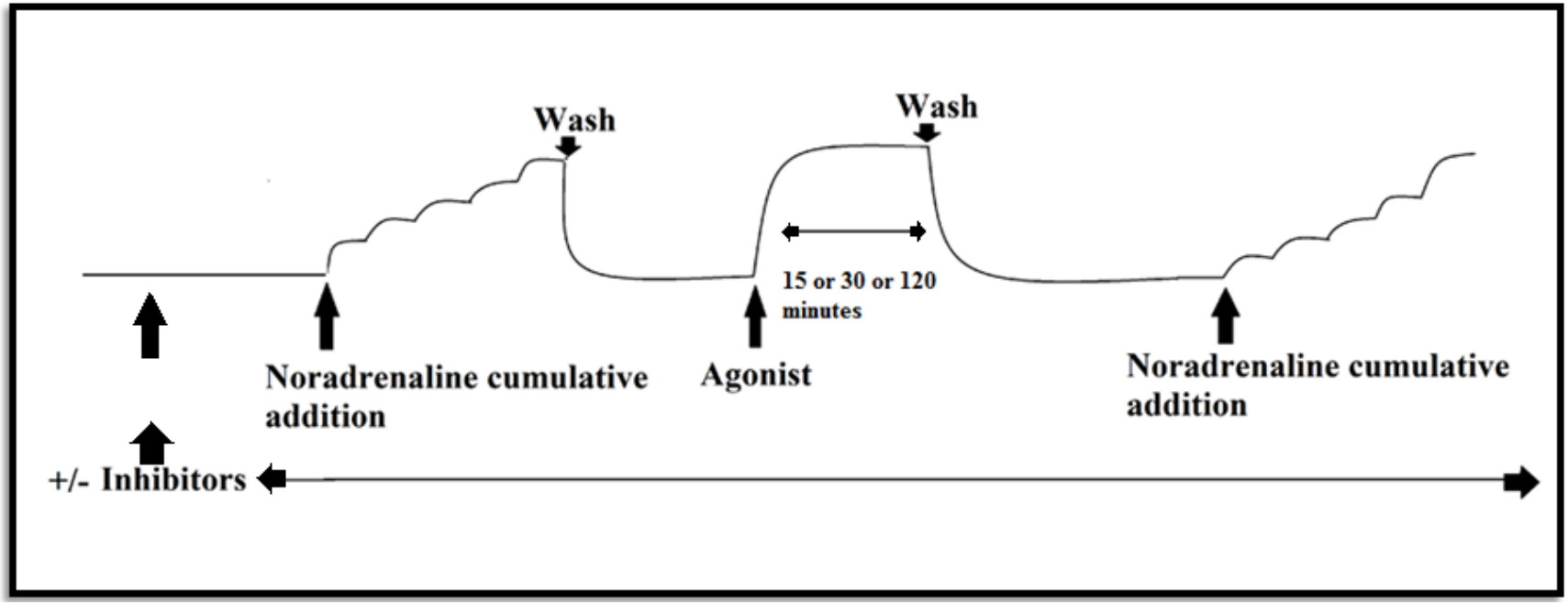
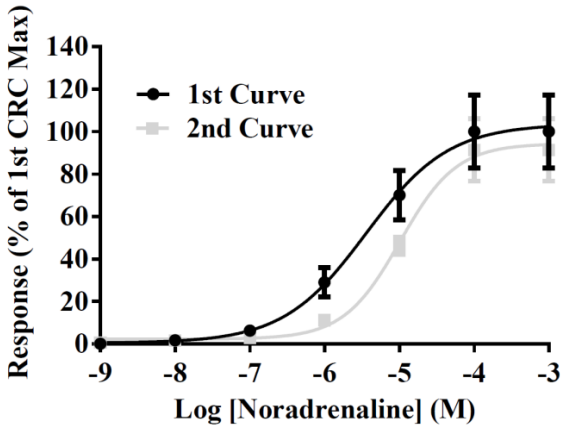


Figure 6.1 Experimental protocol for elucidating desensitisation of α_1-adrenoceptor-mediated responses in the urethral tissues and pathways associated with the desensitisation.

A



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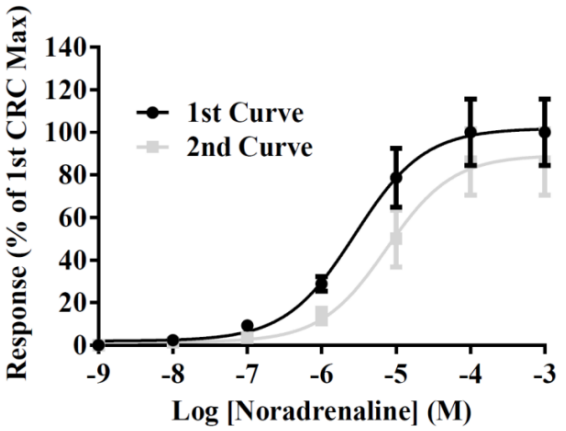

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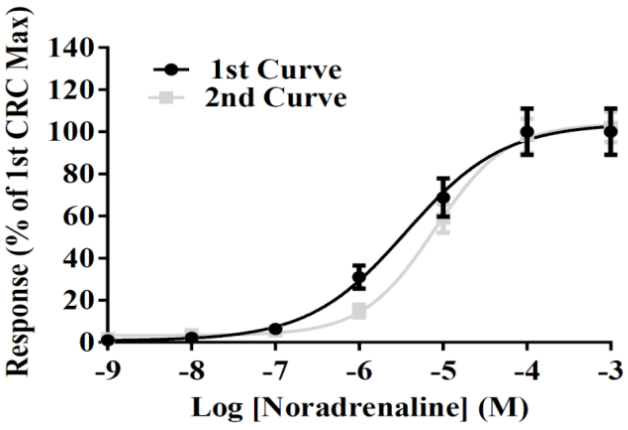


Figure 6.2 Control experiments, first and second concentration-response curves of the urethral tissues to noradrenaline, with (A) 15 minutes, (B) 30 minutes and (C) 120 minutes incubation with Krebs solution only between contraction curves. n=6-8.

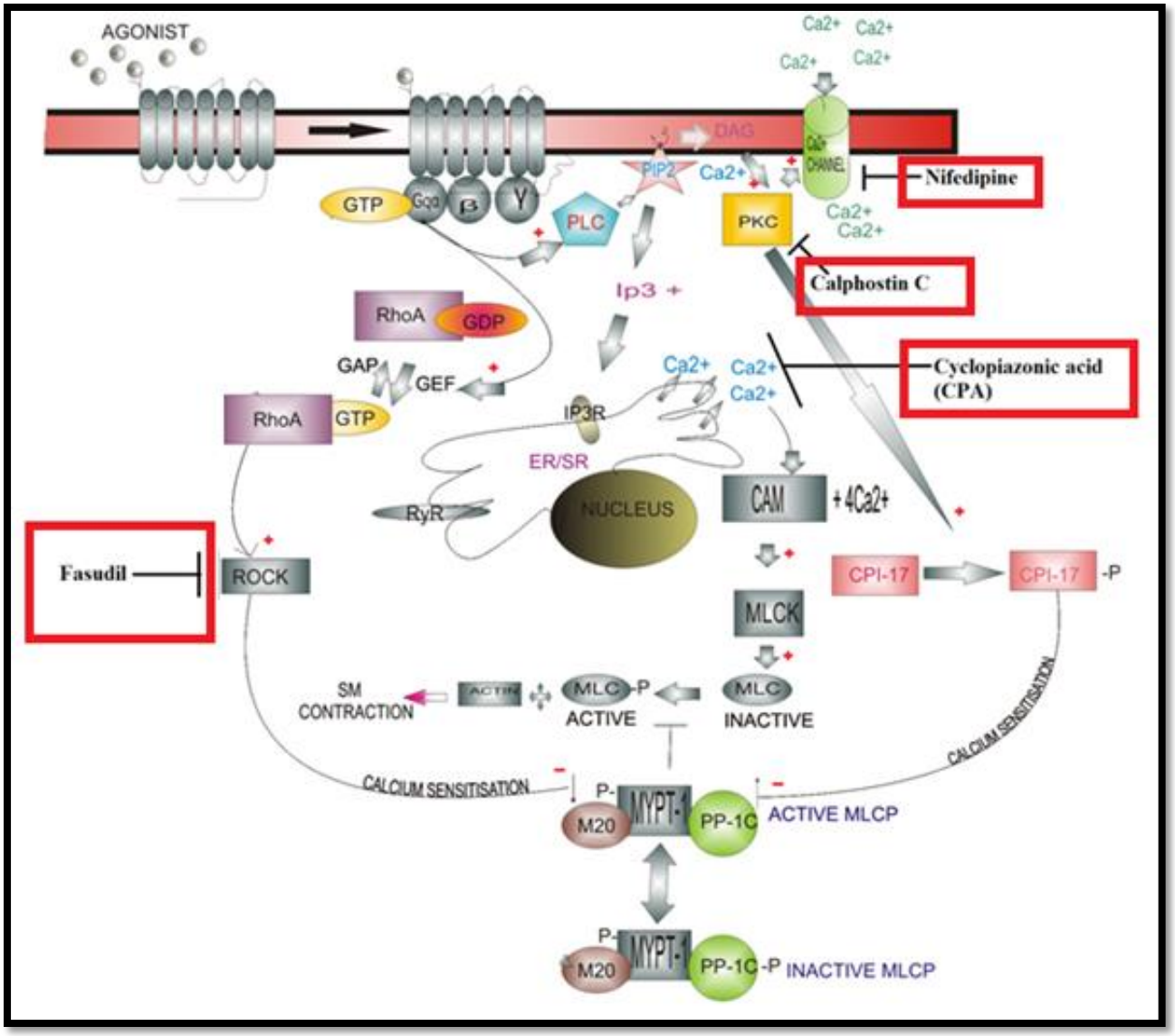


Figure 6.3 Intracellular signalling pathways inhibited to allow analysis of desensitisation of α_1-adrenoceptor mediated responses. Fasudil blocks Rho kinase (ROCK), nifedipine blocks Ca^{2+} influx via the L-type Ca^{2+} channels, calphostin C blocks protein kinase C whilst cyclopiazonic acid blocks sarcoplasmic reticulum release of Ca^{2+}.

6.3 RESULTS

Maintenance of tone following α_1-adrenoceptor stimulation

Following addition of an equi-efficient concentration of agonist (noradrenaline, A61603 or phenylephrine), maximum contractile responses of urethral tissues were recorded within 5-10 minutes (Figure 6.4). The maximal contractile responses were similar for all agonists (Table 6.1). After reaching the maximum contractile response, there was a gradual decline in tone over the duration of the experiment (120 minutes) as shown in Figure 6.4. Responses to noradrenaline rapidly declined during the first 60 minutes and completely by 120 minutes, for phenylephrine tone steadily declined over 120 minutes to 29.0±12.9% of the maximal. In contrast, tone to A61603 slowly declined, and 61.4±7.7% of the tone was maintained at 120 minutes (Table 6.1).

	Maximum Response (mN)	Tone remaining (mN) at 120 minutes	Reduction in tone by 120 minutes (%)
Noradrenaline (10µM)	219.4±22.4	**-12.8±16.5******	105.8±14.6%
Phenylephrine (10µM)	184.2±30.6	**53.4±23.7****	71.0±14.6%
A61603 (30nM)	192.0±18.5	**118.0±14.8***	**38.5±3.0%++**
N	5	5	5

Table 6.1 Maximum responses and tone remaining after 120 minutes following application of noradrenaline (10µM), phenylephrine (10µM) and A61603 (0.03µM) in porcine urethra.*p<0.05, **p<0.01 and ****p<0.0001 vs. maximum responses (Student's t-test). ++ p<0.01 vs. reduction of noradrenaline tone at 120 minutes (one-way ANOVA, with Tukey post-hoc test); n=5.

The role of the urothelium/lamina propria in the maintenance of urethral tone

In separate experiments, using A61603 and phenylephrine, the role of the urothelium/LP in tone maintenance was investigated. Maximum contractions to A61603 and phenylephrine were greater in tissues without urothelium/LP compared to the tissues with urothelium/LP (Table 6.2; Figure 6.5). Contractile responses to phenylephrine decreased significantly over the 120 minutes period, and by a similar amount in tissues with or without urothelium/LP.

For A61603, the percentage of tone remaining at 120 minutes for urethral tissues with urothelium/LP was significantly greater than for those without the urothelium/LP.

	Phenylephrine		A61603	
	- urothelium/LP	**+ urothelium/LP**	**- urothelium/LP**	**+ urothelium/LP**
Max response (mN)	238.0±38.6	127.5±36.4	285.2±60.3	149.7±17.0
Tone remaining at 120 minutes (mN)	**38.7±22.9****	**34.5±6.0***	**61.7±20.3****	**92.4±12.9***
Reduction in tone at 120 minutes (%)	83.8±9.6%	72.9±4.7%	78.4±7.1%	**38.3±8.6%##**
N	6		6	

Table 6.2 Mean (±SEM) contractile responses for phenylephrine and A61603 in urethral tissues with or without urothelium/LP.*p<0.05, **p<0.01 vs. maximum responses. ##p<0.01 vs. percentage reduction in tone to A61603 at 120 minutes for tissues without urothelium/LP (Student's t-test). LP= lamina propria.

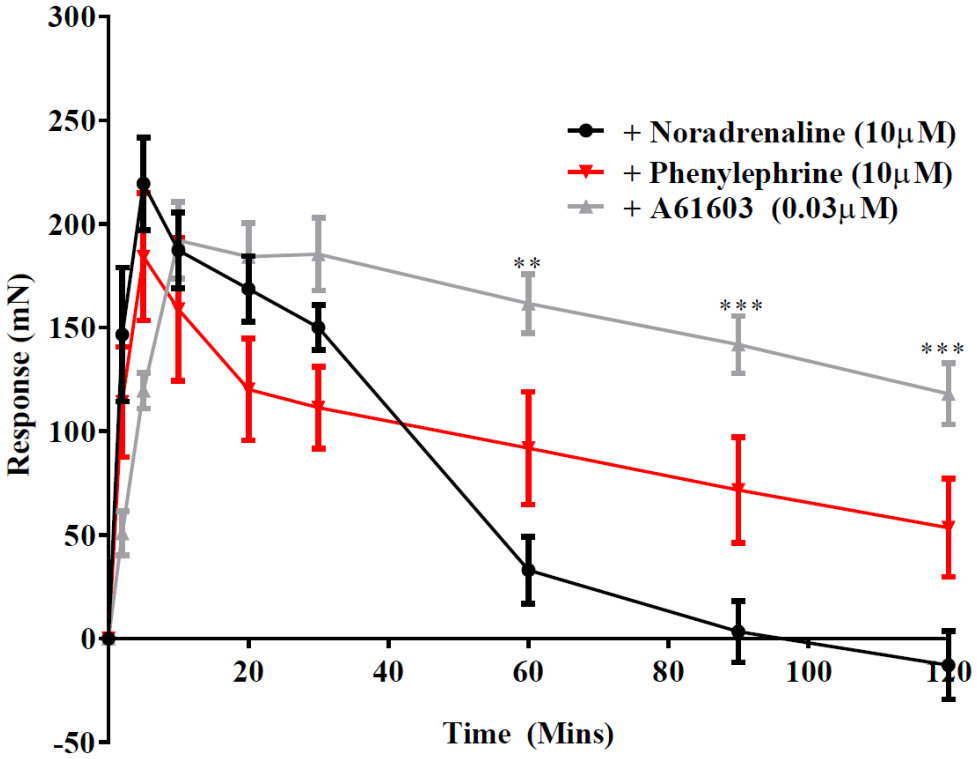


Figure 6.4 Maintenance of tone in urethral tissues with urothelium/lamina propria following exposure to a single concentration of noradrenaline, phenylephrine and A61603 over 120 minutes **p<0.01, ***p<0.001 vs. responses to noradrenaline contraction (one-way ANOVA, with Tukey post-hoc test); n=5.

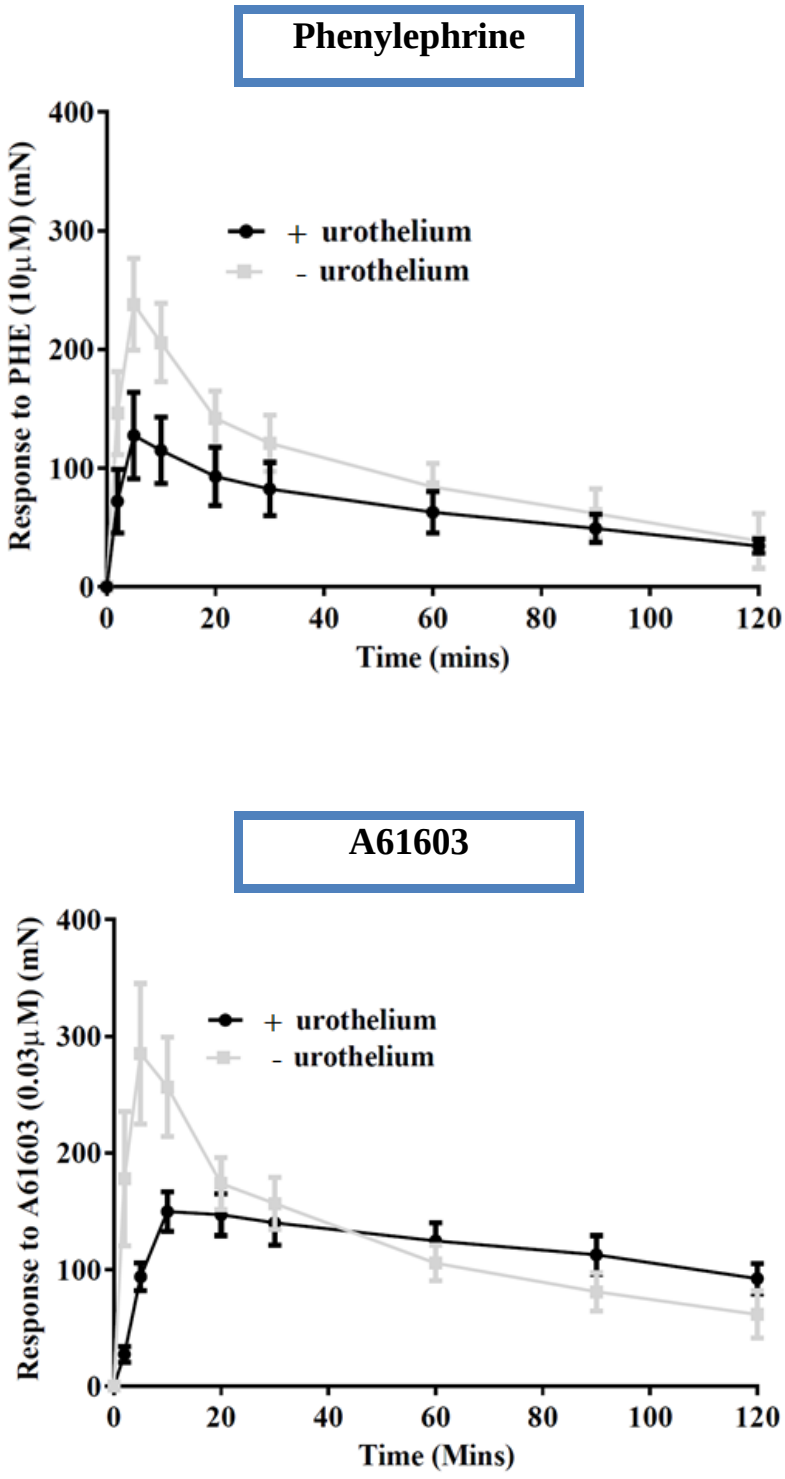


Figure 6.5 Maintenance of tone of urethral tissues with or without urothelium/lamina propria to phenylephrine and A61603 over 120 minutes. n=6. PHE= phenylephrine.

The role of Rho kinase and prostaglandins in urethral tone maintenance

Urethral tissues with urothelium/LP were incubated with fasudil (10μM) or indomethacin (10μM), for 30 minutes before contraction to A61603 (0.03μM) or phenylephrine (10μM), to inhibit Rho kinase and cyclooxygenase activity respectively. Rho kinase inhibition did not significantly reduce the maximum responses of the urethral tissues to phenylephrine compared to controls, as shown in Table 6.3. The tone at 60 minutes and 90 minutes was significantly less than in controls (Figure 6.6), however, the tone over the 120 minutes experimental period was similar to that of the control tissues.

	Phenylephrine		**A61603**	
	Control	**+ Fasudil (10μM)**	**Control**	**+ Fasudil (10μM)**
Max response (mN)	235.2±31.4	168.1±30.0	287.3±19.9	**115.5±30.0****
Tone remaining at 120 minutes (mN)	58.3±7.3	46.4±5.0	243.0±32.1	**94.5±30.2***
Reduction in tone at 120 minutes (%)	75.2±3.1	72.4±3.0	15.4±11.2	18.2±26.1
n	7		4	

Table 6.3 Mean (±SEM) maximum contractile responses and percentage reduction in the responses of urethral tissues after 120 minutes exposure to A61603 (0.03μM) or phenylephrine (10μM) (+/-Rho kinase inhibitor (fasudil)). *p<0.05 and **p<0.01 vs. control responses (Student's t-test).

However, inhibition of Rho kinase did significantly reduce the maximum responses of the urethral tissues to A61603 ($p<0.01$, Table 6.3; Figure 6.6). The percentage difference in maximum contractions between tissues treated with fasudil vs. control tissues was 60.0±16.7%. For A61603 tone was maintained to a similar degree in the presence or absence of fasudil, with reduction in tone by 120 minutes being similar, although the final tone was significantly less in tissues treated with fasudil.

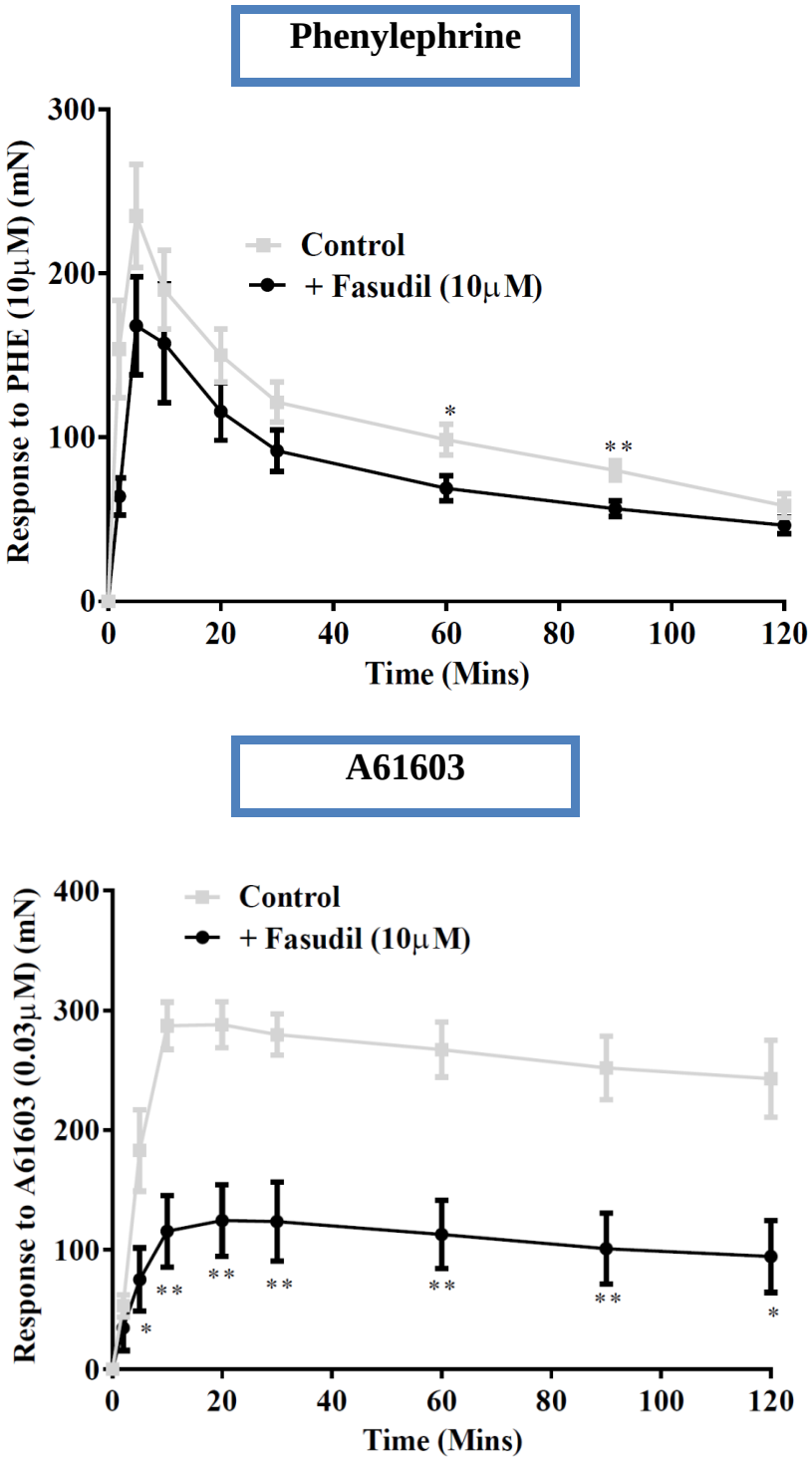


Figure 6.6 Mean (±SEM) contractile responses of the urethral tissues over 120 minutes to phenylephrine and A61603 in the presence or absence of the Rho kinase inhibitor fasudil. n=4-7. *p<0.05, **p<0.01 vs. control responses (Student's t-test). PHE= phenylephrine.

Effect of cyclooxygenase inhibition on urethral tone maintenance

Incubation with indomethacin (10µM) reduced the maximum contractions to phenylephrine and A61603 (Table 6.4). However, neither reduction in maximum responses was statistically significant. Incubation with indomethacin (10µM) had no significant effect on the maintenance of tone following exposure to phenylephrine. However, indomethacin did result in a greater reduction in tone to A61603 at 120 minutes, vs controls, and final tone was significantly less than in controls (Figure 6.7).

	Phenylephrine		**A61603**	
	Control	**+ Indomethacin (10µM)**	**Control**	**+ Indomethacin (10µM)**
Max response (mN)	225.7±35.4	167.2±41.8	287.3±19.9	202.8±66.1
Tone remaining at 120 minutes (mN)	53.3±6.4	47.0±8.9	243.0±32.0	**128.0±33.1***
Reduction in tone at 120 minutes (%)	76.4±2.8	71.9±5.3	15.4±11.2	36.9±16.3
n	6		4	

Table 6.4 Mean (±SEM) maximum contractile responses and percentage change in responses to phenylephrine and A61603 over 120 minutes in the absence or presence of indomethacin. *p<0.05 vs control responses (Student's t-test).

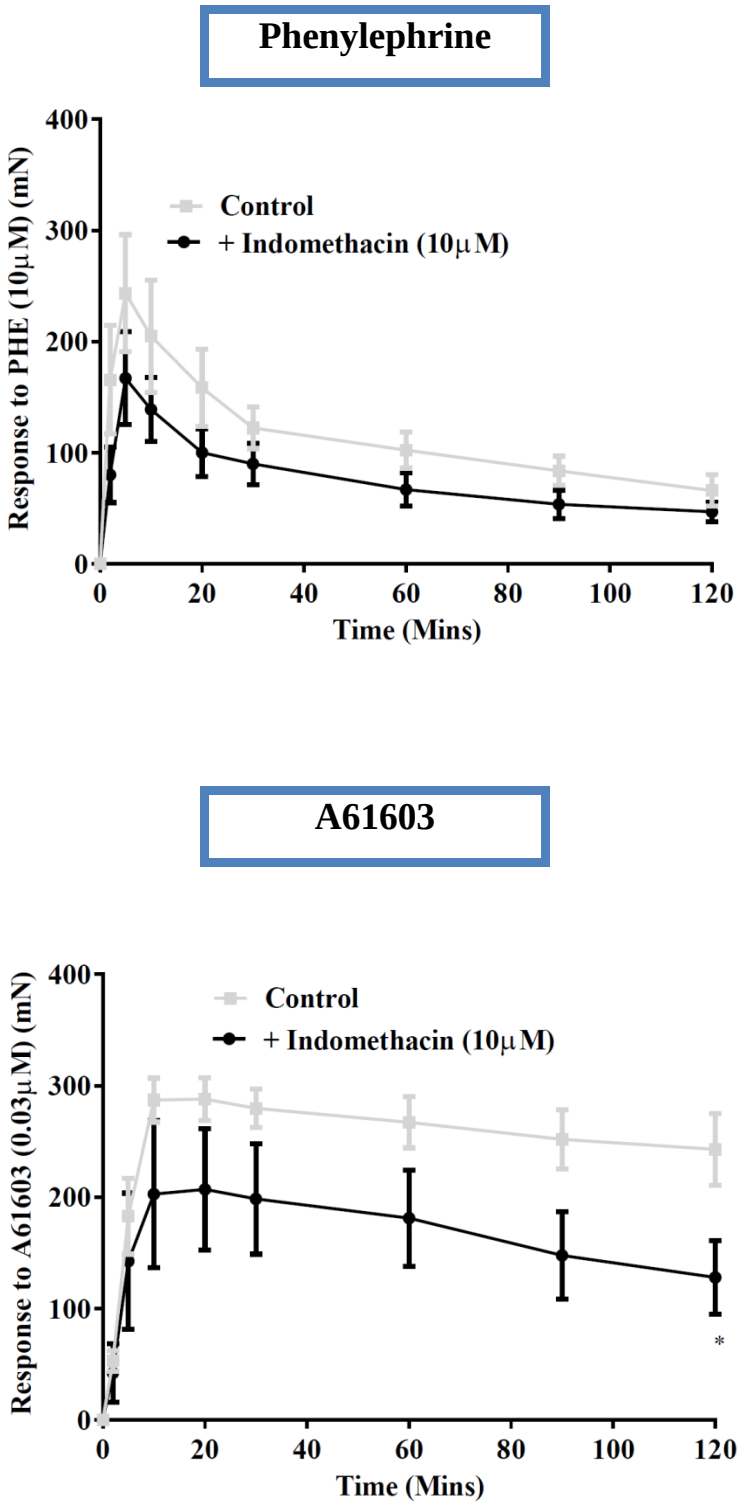


Figure 6.7 Mean (± SEM) contractile responses of the urethral tissues to phenylephrine and A61603 over 120 minutes in the presence or absence of indomethacin (10μM). n=4-6. *p<0.05 vs. control responses (Student's t-test).

Desensitisation of α_1-adrenoceptor-mediated responses

Maximum contractile responses to noradrenaline after incubation with an EC_{50} concentration of noradrenaline (7μM) for 15 minutes (Table 6.5A; Figure 6.8), 30 minutes (Table 6.5B; Figure 6.9) or 120 minutes (Table 6.5C; Figure 6.10) were similar to the maximum responses before incubation. No desensitisation was observed. The pEC_{50} values for noradrenaline before and after incubation with noradrenaline were similar, except for tissues incubated for 120 minutes ($p<0.01$), where the noradrenaline curves were shifted rightward significantly (Figure 6.10).

In contrast, maximum contractile responses of urethral tissues to noradrenaline after incubation with an EC_{50} concentration of phenylephrine (4μM) or A61603 (0.03μM) were significantly desensitised (Table 6.5). Both phenylephrine and A61603 (15, 30 and 120 minutes) triggered desensitisation of urethral responses (Figure 6.8; Figure 6.9; Figure 6.10). The pEC_{50} values for noradrenaline recorded before and after incubation with phenylephrine (4μM) and A61603 (0.03μM) were unchanged by 15 and 30 minutes incubation with phenylephrine. However, for 120 minutes incubation with phenylephrine, the pEC_{50} values for noradrenaline were significantly reduced ($p<0.05$) and the concentration-response curves shifted rightward (Figure 6.10).

A (15 minutes incubation)

	Control	+ Noradrenaline (7µM)	Control	+ Phenylephrine (4µM)	Control	+ A61603 (0.03µM)
Max Response (mN)	137.7±20.8	122.8±19.8	150.0±12.6	**104.0±17.7****	187.9±24.8	**103.6±15.8***
pEC_{50}	5.0± 0.1	5.0± 0.1	5.2±0.1	5.1±0.1	5.2±0.2	5.2± 0.1
Reduction in max (%)	10.8±6.0		30.7±11.8		58.4±17.8	
n	9		10		7	

B (30 minutes incubation)

	Control	+Noradrenaline (7µM)	Control	+Phenylephrine (4µM)	Control	+ A61603 (0.03µM)
Max Response (mN)	214.0±47.3	182.0±36.8	223.8±34.6	**157.2±24.3***	349.0±63.8	**176.1±17.5***
pEC_{50}	5.1±0.1	5.1±0.1	5.1±0.1	5.1±0.1	5.0±0.1	5.2± 0.1
Reduction in max (%)	15.0±17.2		29.8±10.8		49.5±5.0	
n	**4**		**6**		**9**	

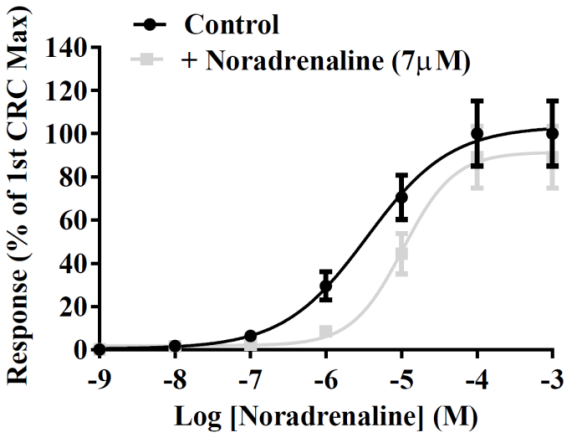
C (120 minutes incubation)

	Control	+ Noradrenaline (7µM)	Control	+ Phenylephrine (4µM)	Control	+ A61603 (0.03µM)
Max Response (mN)	116.8±23.0	95.9±17.7	232.9±21.2	**182.4±16.1***	185.6±19.7	**102.8±19.5***
pEC_{50}	5.3±0.1	**4.9±0.1****	5.2±0.1	**5.0±0.1***	5.4±0.2	5.1±0.1
Reduction in max (%)	17.9±15.2		21.7±6.9		44.6±10.5	
n	**9**		**7**		**5**	

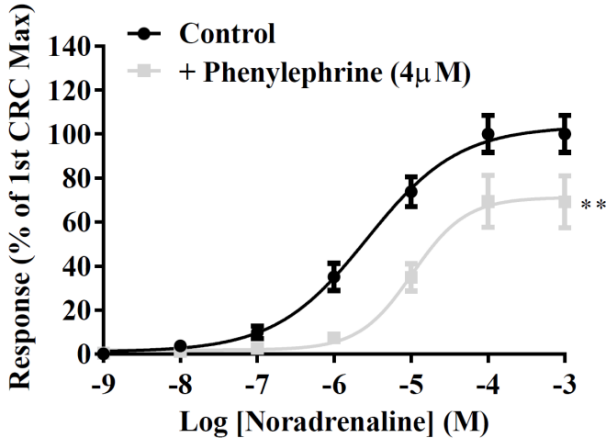
Table 6.5 Mean (±SEM) maximum contractile responses and pEC_{50} values for noradrenaline responses before and after incubation with noradrenaline (7µM), phenylephrine (4µM) or A61603 (0.03µM) for (A) 15 minutes, (B) 30 minutes and (C) 120 minutes. *p<0.05, **p<0.01 vs. control responses (Paired Student's t-test).

15 minutes incubation

Noradrenaline



Phenylephrine



A61603

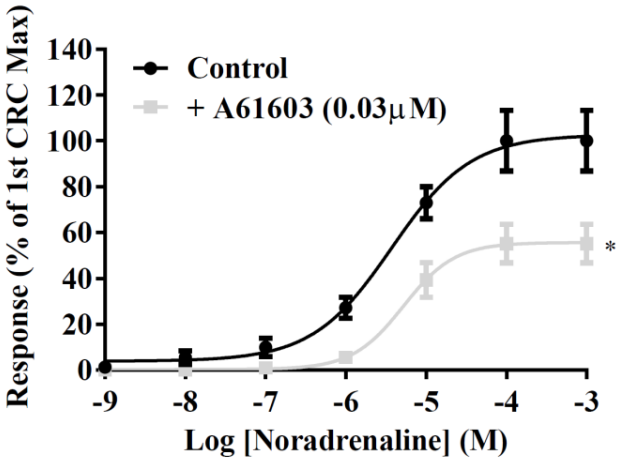
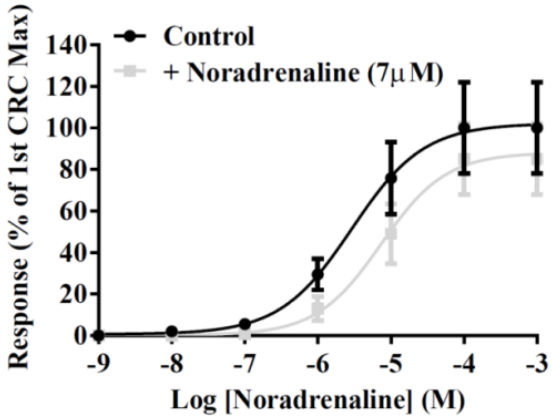


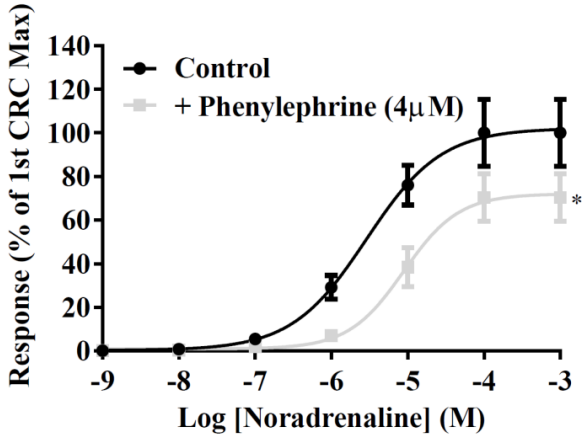
Figure 6.8 Mean (± SEM) concentration-response curves (CRC) to noradrenaline before and after 15 minutes incubation with noradrenaline, phenylephrine and A61603. n=7-10, *p<0.05, **p<0.01 vs. control responses (Paired Student's t-test).

30 minutes incubation

Noradrenaline



Phenylephrine



A61603

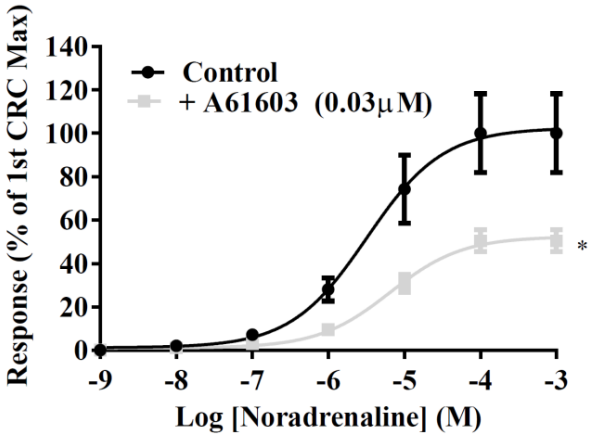
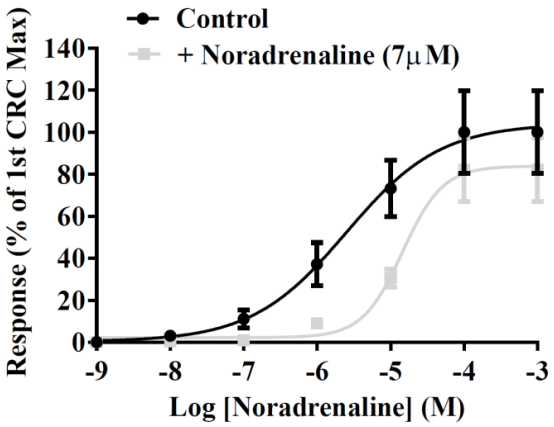


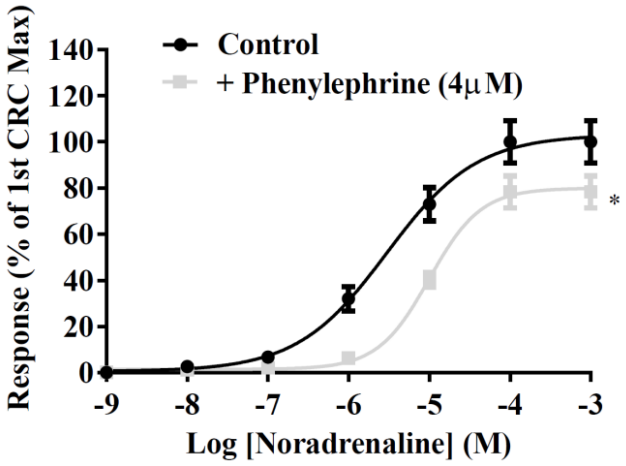
Figure 6.9 Mean (± SEM) concentration-response curves (CRC) to noradrenaline before and after 30 minutes incubation with noradrenaline, phenylephrine and A61603. n=4-9; *p<0.05 vs. control responses (Paired Student's t-test).

120 minutes incubation

Noradrenaline



Phenylephrine



A61603

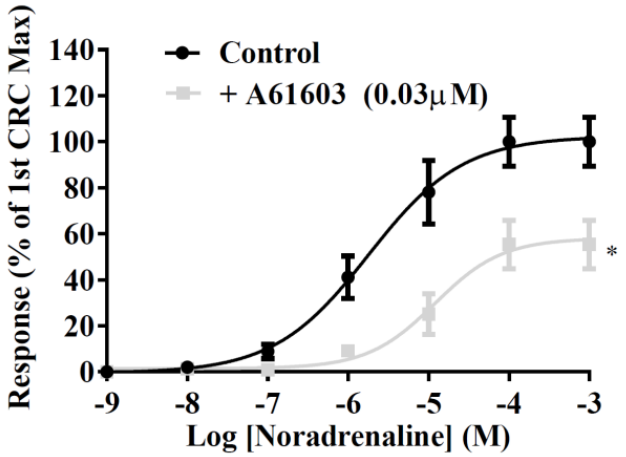


Figure 6.10 Mean (± SEM) concentration-response curves (CRC) of the urethral tissues to noradrenaline before and after 120 minutes incubation with noradrenaline, phenylephrine and A61603. n=5-9. *p<0.05 vs. control responses (Paired Student's t-test).

The role of Ca^{2+}, Rho kinase, and PKC in desensitisation of α_1-adrenoceptor responses

Nifedipine (1μM) prevented the desensitisation of noradrenaline responses induced by 15 minutes incubation with the EC_{50} concentration of A61603 ($p<0.05$), but not the desensitisation induced by phenylephrine (Table 6.6; Figure 6.11). The pEC_{50} values for noradrenaline were similar in all tissues.

	Without Nifedipine		*With Nifedipine*		*Without Nifedipine*		*With Nifedipine*	
	Control	**+ A61603**	**Control**	**+ A61603**	**Control**	**+ PHE**	**Control**	**+ PHE**
Max response (mN)	187.9±24.8	**103.6±15.8***	81.4±13.5	69.5±20.2	150.0±12.6	**104.0±17.7****	133.5±25.3	**93.3±19.6****
pEC_{50}	5.2±0.2	5.2±0.1	5.5±0.1	5.1±0.2	5.2±0.1	5.1±0.1	5.1±0.1	5.0±0.0
Percentage reduction in max (%)	44.9±8.4		14.6±14.2		30.7±11.8		30.1±6.4	
n	7		5		10		10	

Table 6.6 Mean (±SEM) maximum contractile responses and pEC_{50} values for noradrenaline before and after 15 minutes incubation with phenylephrine or A61603, in the presence or absence of nifedipine (1μM). *p<0.05, **p<0.01 vs. control responses (Paired Student's t-test). PHE= phenylephrine.

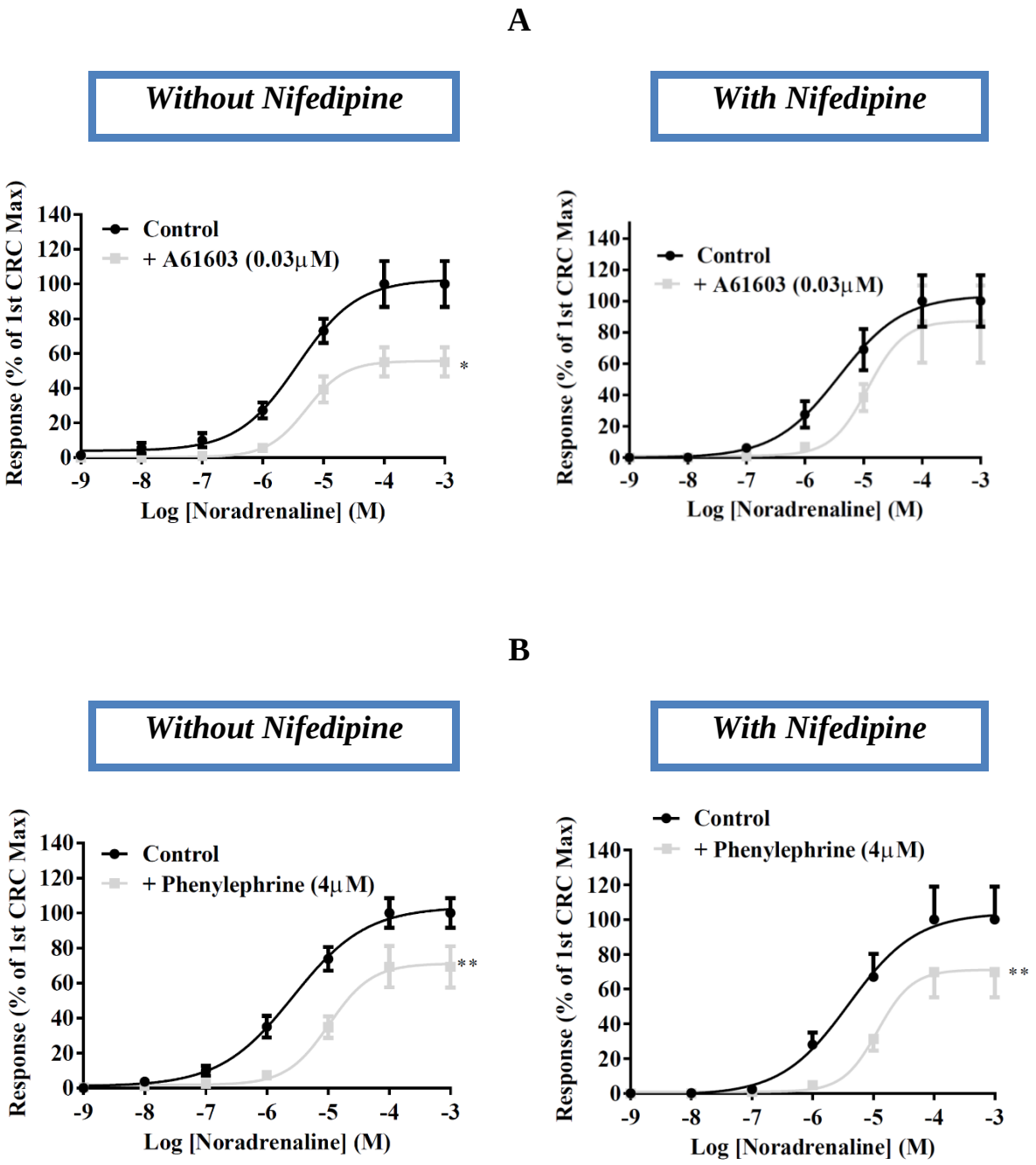


Figure 6.11 Mean (±SEM) concentration-response curves of urethral tissues to noradrenaline with or without prior incubation (15 minutes) with (A) A61603 (0.03μM) or (B) phenylephrine (4μM). The experiments were carried out in the presence or absent of nifedipine (1μM). n=5-10. *p<0.05, **p<0.01 vs. control responses (Paired Student's t-test).

In the presence of CPA (10µM) to inhibit Ca^{2+} release from the sarcoplasmic reticulum maximum contractile responses to cumulative concentrations of noradrenaline after incubation with the EC_{50} concentration of A61603 or phenylephrine remained significantly desensitised ($p<0.05$; Table 6.7, Figure 6.12). The pEC_{50} values for noradrenaline were similar.

Fasudil (10µM) prevented the desensitisation of the maximum responses to noradrenaline induced by A61603 and phenylephrine (Table 6.8; Figure 6.13). The pEC_{50} values for noradrenaline were not affected by fasudil (10µM).

Moreover, calphostin C (1µM) prevented the desensitisation of the maximum responses to noradrenaline induced by A61603, but not the desensitisation induced by phenylephrine (Table 6.9; Figure 6.14). The pEC_{50} values for noradrenaline were not affected by calphostin C (1µM).

	Without CPA		***With CPA***		***Without CPA***		***With CPA***	
	Control	**+ A61603**	**Control**	**+ A61603**	**Control**	**+ PHE**	**Control**	**+ PHE**
Max response (mN)	187.9±24.8	**103.6±15.8***	256.0±50.1	**134.3±38.1***	150.0±12.6	**104.0±17.7****	202.6±46.3	**108.8±15.3***
pEC_{50}	5.2±0.2	5.2±0.1	5.2±0.2	5.2±0.2	5.2±0.1	5.1±0.1	5.2±0.1	5.1±0.1
Reduction in max (%)	44.9±8.4		47.6±13.9		30.7±11.8		46.3±15.6	
n	7		4		10		9	

Table 6.7 Mean (±SEM) maximum contractile responses and pEC_{50} values for noradrenaline with or without 15 minutes incubation with phenylephrine (4µM) or A61603 (0.03µM). The experiments were carried out in the presence or absence of cyclopiazonic acid (CPA) (10µM). *$p<0.05$, **$p<0.01$ vs. control responses (Paired Student's t-test). PHE= phenylephrine.

	Without Fasudil		*With Fasudil*		*Without Fasudil*		*With Fasudil*	
	Control	**+ A61603**	**Control**	**+ A61603**	**Control**	**+ PHE**	**Control**	**+ PHE**
Max response (mN)	187.9±24.8	**103.6±15.8***	182.8±36.5	180.0±31.2	150.0±12.6	**104.0±17.7****	204.9±43.2	202.3±46.3
pEC_{50}	5.2±0.2	5.2±0.1	4.9±0.0	5.0±0.0	5.2±0.1	5.1±0.1	4.9±0.0	4.9±0.0
Reduction in max (%)	44.9±8.4		**1.5±9.6##**		30.7±11.8		**1.3±2.3#**	
n	7		4		10		9	

Table 6.8 Mean (±SEM) maximum contractile responses and pEC_{50} values for noradrenaline with or without 15 minutes prior incubation with agonists in the presence or absence of fasudil (10μM). *p<0.05, **p<0.01 vs. control responses (Paired Student's t-test); #p<0.05, ##p<0.01 vs. percentage reduction in maximum without fasudil (Student's t-test). PHE= phenylephrine.

	Without calphostin C		*With calphostin C*		*Without calphostin C*		*With calphostin C*	
	Control	**+ A61603**	**Control**	**+ A61603**	**Control**	**+ PHE**	**Control**	**+ PHE**
Max response (mN)	187.9±24.8	**103.6±15.8***	254.3±62.2	262.9±71.3	150.0±12.6	**104.0±17.7****	151.0±39.2	**81.0±25.2***
pEC_{50}	5.2±0.2	5.2±0.1	5.1±0.1	5.1±0.1	5.2±0.1	5.1±0.1	5.2±0.2	5.0±0.0
Reduction in max (%)	44.9±8.4		**3.4±4.7###**		30.7±11.8		46.4±13.5	
n	7		8		10		5	

Table 6.9 Mean (±SEM) maximum contractile responses and pEC_{50} values for noradrenaline with or without 15 minutes prior incubation with agonists in the presence or absence of calphostin C (1μM). *p<0.05, **p<0.01 vs. control responses (Paired Student's t-test); ###p<0.001 vs. percentage reduction in maximum for tissues without calphostin C (Student's t-test). PHE= phenylephrine.

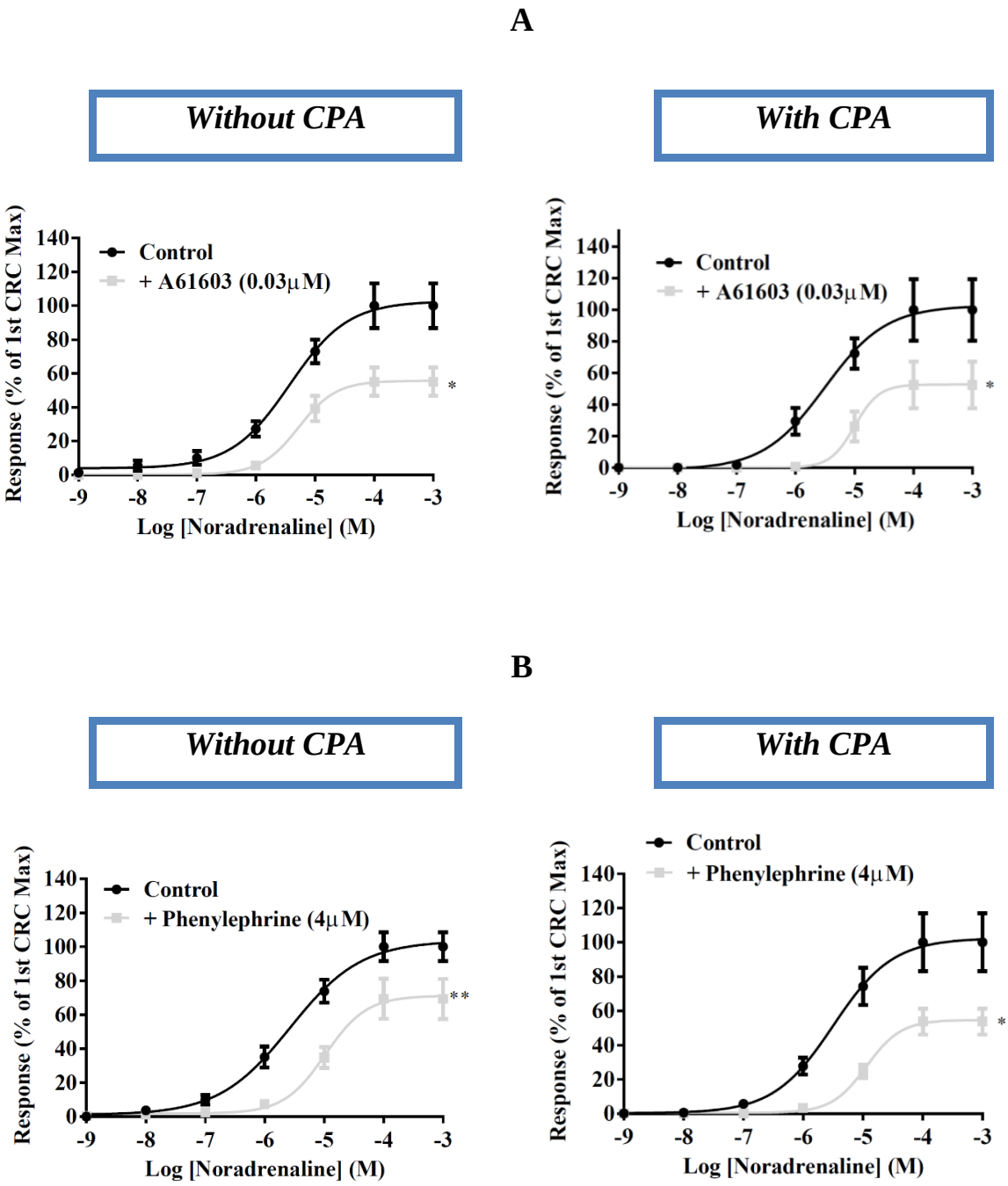


Figure 6.12 Mean (±SEM) concentration-response curves (CRC) of the urethral tissues to noradrenaline with or without prior 15 minutes incubation with agonist. The experiments were carried out in the presence or absence of cyclopiazonic acid (CPA) (10μM). (A) A61603 (0.03μM) or (B) phenylephrine (4μM), n=4-10; *p<0.05, **p<0.01 vs. control responses (Paired Student's t-test).

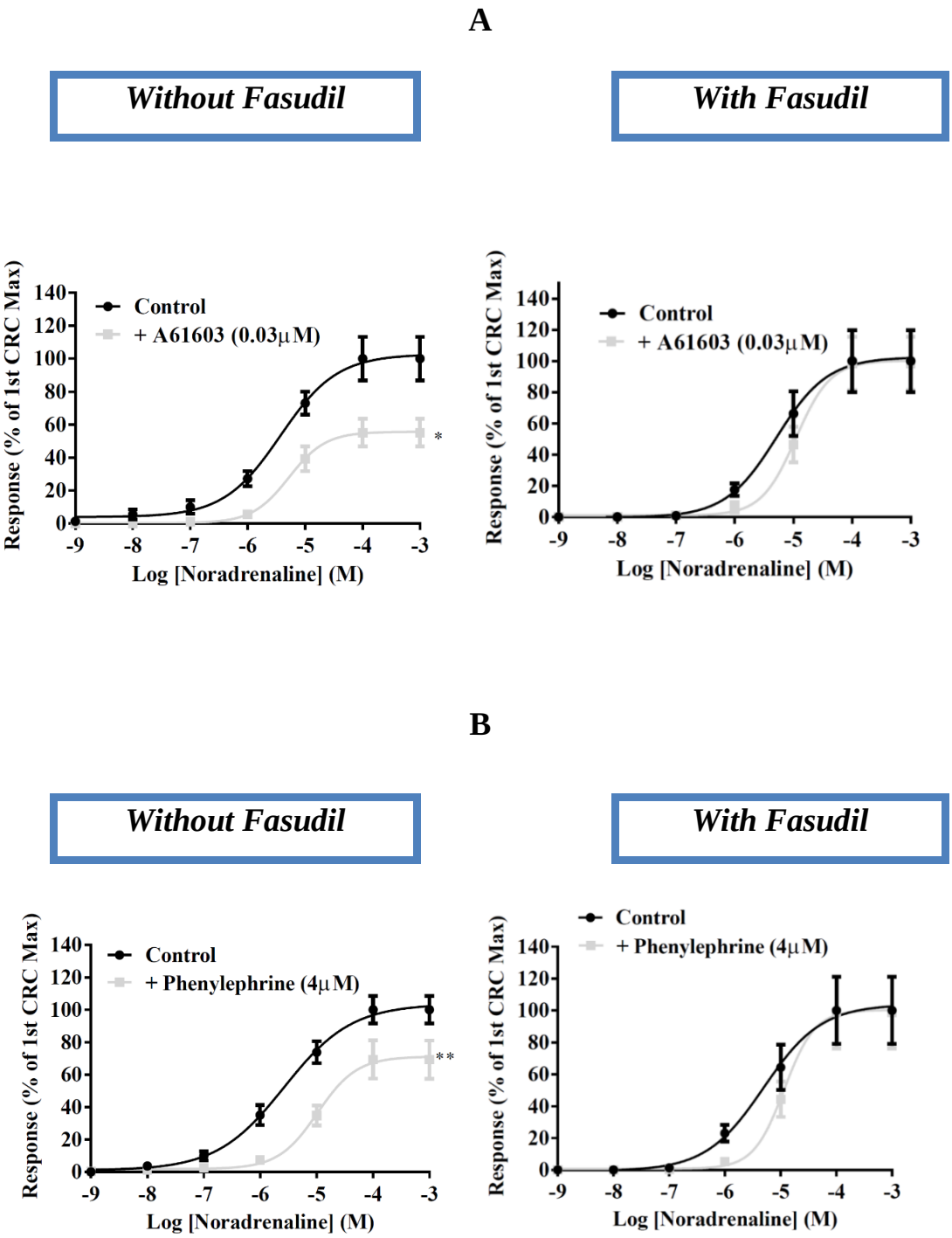
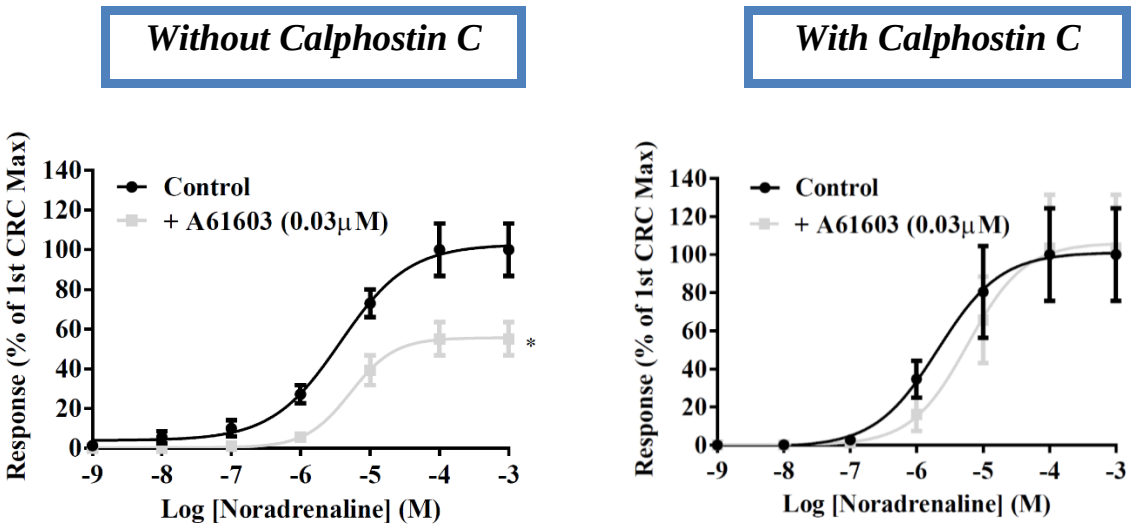


Figure 6.13 Mean (±SEM) concentration-response curves (CRC) to noradrenaline before and after 15 minutes incubation with agonists in the presence or absence of fasudil (10μM). (A) A61603 (0.03μM) or (B) phenylephrine (4μM); n=4-10. *p<0.05, **p<0.01 vs. control responses (Paired Student's t-test).

A



B

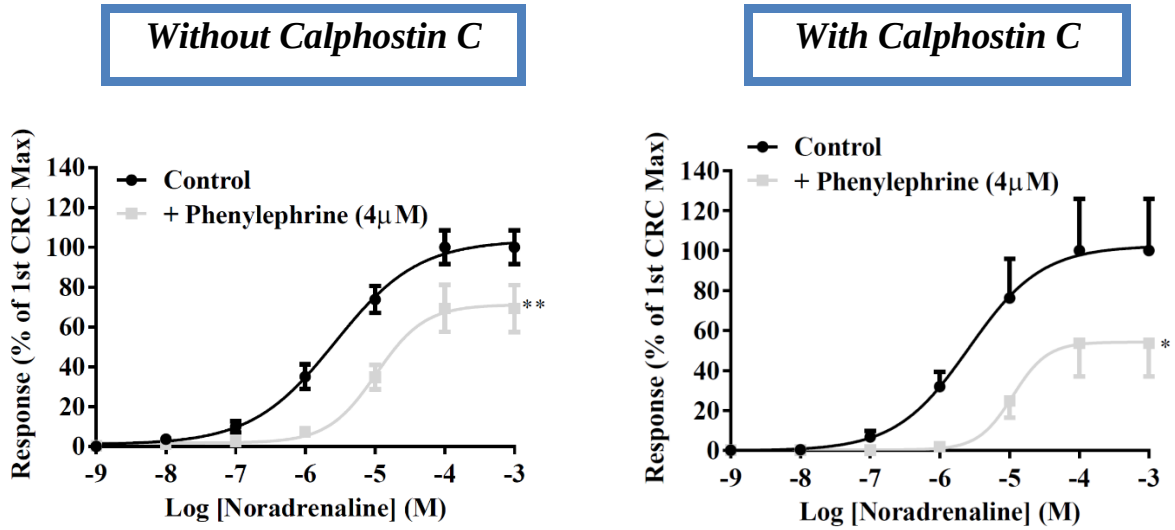


Figure 6.14 Mean (±SEM) concentration-response curves (CRC) to noradrenaline before and after 15 minutes incubation with agonists in the presence or absence of calphostin C (1µM). (A) A61603 (0.03µM) or (B) phenylephrine (4µM), n=5-10; *p<0.05, **p<0.01 vs. control responses (Paired Student's t-test).

6.4 DISCUSSION

The data in this study revealed that functionally there is agonist-mediated desensitisation of urethral tissue responses during 2 hours incubation with a single dose of an α_1-agonist. We showed that noradrenaline-induced tone declined significantly during a 120-minute incubation, and tissues incubated with phenylephrine retained a small amount of tone, whilst A61603-induced responses reduced only slightly after 120 minutes incubation. Thus urethral tissues maintained tone to A61603 during these experiments. Furthermore, experiments confirmed that the maintenance of tone was not associated with Rho kinase or prostaglandins.

Can maintenance of tone following stimulation with single α_1-adrenoceptor agonist concentration be demonstrated in the urethral tissue?

Exposure of α_1-ARs to an agonist for an extended period will induce desensitisation (Akinaga et al., 2013). Such a phenomenon has been shown for the α_1-ARs in HEK-293 cells, vascular smooth muscle and vas deferens of the rat (Akinaga et al., 2013). This report showed desensitisation to repeated addition of agonist. Reports showing tone maintenance and desensitisation during a prolonged exposure to a single dose of agonist are limited and particularly in the urethra. Chess-Williams & Bagot, (2005) showed that during a 120-minute incubation with methoxamine or A61603, porcine urethral tissues contracted initially but then tension declined by 25-60% during the incubation period. In this study, we further explored the maintenance of urethral tone to a single dose of noradrenaline and phenylephrine in addition to A61603. Practically, during treatment for stress urinary incontinence with α_1-AR agonist, the drugs will be expected to be in contact with the ARs for an extended period. Thus, it is imperative to know if the long-term exposure to the α_1-AR-agonist is likely to continue to affect the urethral luminal pressure.

During the 120 minutes incubation, tissues contracted initially but then tension declined during the incubation with the α_1-AR agonists. Also, the maintenance of tone was proportional to agonist specificity, with noradrenaline (general AR agonist) tone desensitising the greatest, and A61603 (α_{1A}-AR agonist) showing the least decline in tone for tissues with urothelium. When the urethral tissues attained the maximum contractions, a sustained response was developed in tissues contracted to A61603, while tissues contracted to

noradrenaline and phenylephrine diminished significantly. It was glaring that A61603-induced responses were more resistant to loss of tone compared to the other agonists.

A possible explanation for the difference in maintenance of tone observed between A61603 and the other agonists is a difference in intracellular signalling pathways. For example, the contribution of Rho kinase and PKC to Ca^{2+} sensitization pathway may differ, as shown by Shahab et al., (2012a) in human detrusor and they may be regulated differently, as shown by Hayashi et al., (2016) in human detrusor. It is likely that the mechanism of tone maintenance differs for the agonists. In the previous chapter, we showed that A61603-mediated urethral contraction depends largely on Ca^{2+} sensitization, compared to the contraction induced by noradrenaline and phenylephrine. Thus, A61603 may increase the Ca^{2+} sensitivity of the urethra. With the ability of A61603 to potently stimulate phosphoinositide hydrolysis (Knepper et al., 1995) and Ca^{2+} sensitization, this may be responsible for the urethral tone to be significantly maintained compared to the other two agonists.

As described earlier, decline in tone may be associated with receptor phosphorylation, desensitisation (Stanasila et al., 2008) and internalisation (Wang et al., 2007). GRK (Karoor & Malbon, 1996) or serine/threonine kinase, PKC may also play a vital role in mediating this decline in urethral tone (Akinaga et al., 2013; Molina-Muñoz et al., 2006).

The resistance to desensitisation of tone with A61603 does not signify the absence of desensitisation. There is the possibility of desensitisation which may be characterised by a prompt recovery and re-sensitisation of α_{1A}-AR as shown by Morris et al., (2004) in Rat-1 fibroblasts cells. Also, an internal pool of α_{1A}-ARs may be maintained and recycled to allow continuous agonist-induced signalling as shown by Morris et al., (2004) in Rat-1 fibroblasts cells. Protein kinase C may in part mediate desensitisation of the α_1-ARs (Akinaga et al., 2013), and phosphorylated GPCR may bypass the destruction in the lysosome. Thus, a reinsertion into the plasma membrane and readiness for another activation as shown for β-AR (Morrison et al., 1996; Pippig et al., 1995).

A closer look at the profile of tone maintenance over 120 minutes revealed that the fall in tension in phenylephrine incubated tissues exhibited a biphasic profile with an initial phase from 5-10 minutes followed by a second phase from 30 to120 minutes, while that of

noradrenaline did not occur evenly over the 120 minutes. The major decline in noradrenaline-induced response occurred within the first 60 minutes. This decline continued throughout the experimental period, and responses at the end of the incubation were below the initial basal level tone. Thus, there is the possibility of downregulation of α_1-ARs in response to noradrenaline. This downregulation of α_1-ARs is initiated by kinases, and may involve destruction or digestions in the lysosome which require gene transcription/translation before a new set of α_1-ARs are expressed (McGraw et al., 1998). However, a comprehensive experiment determining receptor density and location during exposure would be needed to confirm this inference.

Does the urothelium/lamina propria affect maintenance of tone?

Previous studies have investigated the effect of the urothelium/LP on modulation of GPCR mediated responses (Hawthorn et al., 2000; Templeman et al., 2002), but have not examined the modulation of tone in response to prolonged α_1-AR activation. In earlier chapters, we showed that the urothelium/LP inhibits smooth muscle contraction in the urethra. In this chapter, the possibility of the urothelium/LP inhibiting or mediating urethral tone maintenance was investigated. For this study, only phenylephrine and A61603 were used because A61603 is an α_{1A}-AR specific agonist and urethral tone to this agonist was maintained, while phenylephrine is a known α_1-AR agonist previously used in clinical trials for incontinence, and less tone was maintained during the 120 minutes incubation period.

It seems removing the urothelium/LP affects the maximal contraction more but final tone at 120 minutes is not different. Thus, the urothelium/LP does not contribute to maintenance of tone following exposure to α_1-AR agonist**.**

Are Rho kinase and prostaglandins involved in maintenance of urethral tone?

Surprisingly, the addition of fasudil (10μM) had no significant effect on the maintenance of tone following exposure to α_1-AR agonist. The A61603-induced maximum contraction was inhibited by fasudil (10μM) indicating the Rho kinase pathway was involved in mediating contraction as shown by Teixeira et al., (2007) in the rat urethra. However, the maintenance

of tone during incubation with A61603 was not affected. In contrast, inhibition of Rho kinase did not have any significant effect on the maximum contractile response to phenylephrine, and did not affect the decline of tone during incubation with phenylephrine. This data supports the results in chapter 4, where we showed that Ca^{2+} sensitisation contributed more to A61603-mediated responses than phenylephrine-mediated responses. Thus, we concluded that Rho kinase does not contribute to maintenance of tone.

Another possible agent that could be involved in tone maintenance are prostaglandins. If prostaglandins were involved, relaxing prostaglandins such as prostaglandin E_2 may be released and its release may desensitise the EP receptors, thus, affecting maintenance of tone. Alternatively, a contractile prostaglandin e.g. prostaglandin $F_{2\alpha}$ may be released which may counteract the agonist-induced desensitisation. Similar to the result from incubation of tissues with fasudil (10μM), the addition of indomethacin (10μM) had no significant effect on the effects of the urothelium on tone maintenance during α_1-AR-mediated responses indicating prostaglandins did not contribute to tone maintenance.

Thus tone maintenance in response to α_1-AR activation is greater for A61603, followed by phenylephrine, whereas no tone was maintained to noradrenaline. Moreover, neither Rho kinase nor prostaglandins contributed to tone maintenance.

Can α_1-adrenoceptor stimulation desensitise subsequent responses to α_1-adrenoceptor agonist?

Earlier we showed that urethral tissue responses desensitise on exposure to a single dose of α_1-AR agonist. However, a further question was, what happens to the response to the endogenous agonist noradrenaline after prior exposure to synthetic α_1-AR agonists? We thereby tested the effect of prior incubation with α_1-AR agonists on the urethral tissue responses to subsequent noradrenaline addition. Based on the result of the earlier section and earlier reports (Price et al., 2002; Vazquez-Prado et al., 2000), we hypothesised that incubation of urethral tissues with noradrenaline, phenylephrine or A61603 would induce desensitisation of subsequent urethral responses to noradrenaline.

In the present study, incubation of porcine urethra with an EC_{50} concentration of noradrenaline for up to 120 minutes failed to affect subsequent responses to noradrenaline.

This finding agrees with numerous reports showing that α_{1A}-ARs are resistant to noradrenaline-mediated desensitisation (Vazquez-Prado et al., 2000; Cabrera-Wrooman et al., 2010). It is well established that α_{1A}-ARs are less phosphorylated and desensitised on exposure to noradrenaline (Vazquez-Prado et al., 2000; Cabrera-Wrooman et al., 2010) and are less internalised (Cabrera-Wrooman et al., 2010). Akinaga et al., (2013) observed, using HEK-293 cells, rat tail artery and vas deferens, that phosphorylation of α_{1A}-ARs exposed to noradrenaline depends to a large extent on PKC activity and is not followed by desensitisation. Moreover, α_{1A}-ARs undergo delayed internalisation after exposure to noradrenaline (Akinaga et al., 2013). Thus, the lack of significant desensitisation to noradrenaline after prior incubation with noradrenaline may be due to the characteristic of α_1-ARs being less phosphorylated, desensitised and internalise by noradrenaline.

However, phenylephrine and A61603 did cause desensitisation of noradrenaline responses. After incubations for 15, 30 or 120 minutes with EC_{50} concentrations of drug the rank order of desensitisation for the time points was similar, with desensitisation to A61603 greater than for phenylephrine. A61603 desensitised α_1-AR-mediated responses to a greater extent in the urethra at all three-time points. Thus, an agonist with greater specificity for α_{1A}-ARs induced greatest desensitisation to noradrenaline.

The literature regarding the effect of long-term exposure to these different agonists is currently limited. The most frequently used agonist in desensitisation studies of α_1-ARs in addition to noradrenaline is phenylephrine (Wang et al., 2007; Zhao et al., 2015). However, these two agonists are not often used in parallel in the same cells/tissues, so it is arduous to judge whether the effects observed occurred separately of the stimulating agonist used. However, the literature does show that A61603 induced desensitisation of responses in cells, for example in astrocytes (Pankratov & Lalo, 2015). In astrocytes, application of A61603 (100 nM) caused a marked reduction in the response amplitude and led to a period of non-responsiveness (Pankratov & Lalo, 2015).

The similarity of desensitisation responses at the three-time points suggests that desensitisation, phosphorylation and sequestration occurred at an early time point, while the effect was still manifested at 120 minutes. Wang et al., (2007) observed the internalisation of the α_{1A}-ARs as early as 5 minutes after phenylephrine stimulation in HEK-293 cells. An

approximate 40% of surface α_{1A}-ARs were internalised after 5 minutes of stimulation with α_{1A}-AR agonist (oxymetazoline) in the HEK-293 cells (Akinaga et al., 2013). Likewise, Morris et al., (2008) reported receptor signalling and desensitisation of the α_{1A}-ARs within 3-10 minutes in the Rat-1 fibroblasts. Morris et al., (2008) confirmed the desensitisation because these receptors exited the lipid raft and non-raft plasma membrane within this timeline after stimulation with phenylephrine. Also, this result further suggests that the second messenger system and kinases such as PKC, GRKs associated with GPCR phosphorylation and signalling are activated within the first few minutes (Akinaga et al., 2013). Akinaga et al., (2013) reported GRK2-dependent α_{1A}-AR phosphorylation, as early as 5 minutes with α_{1A}-AR agonist. Also, Zhao et al., (2015) reported the activation of PKC, 40 minutes after stimulation of the rat's aorta with phenylephrine.

Greater desensitisations induced by A61603 over phenylephrine at the three-time points may be associated with downstream signalling pathways activated by the respective agonist. For example, in the rat tail artery and vas deferens, Akinaga et al., (2013) showed that oxymetazoline (a partial α_{1A}-ARs), but not phenylephrine or noradrenaline, desensitised responses. Oxymetazoline induced GRK2-mediated phosphorylation of α_{1A}-ARs followed by rapid desensitisation and internalisation (~40% internalisation after 5 minutes of stimulation) (Akinaga et al., 2013). Thus, A61603 may induce greater desensitisation due to greater potential to activate GRK in the urethra.

Moreover, greater desensitisation mediated by A61603 over phenylephrine could also be associated with agonist efficacy. A61603 has a greater efficacy than to phenylephrine (Bagot & Chess-Williams, 2006; Mills et al., 2008). Hence, an agonist with greater efficacy may be able to induce greater desensitisation. The relationship between agonist efficacy and amplitude of desensitisation is well established for the β-AR. For example, salmeterol (lower efficacy β-AR agonist) was associated with lower desensitisation compared with formoterol (Moore et al., 2007; January et al., 1997; 1998) and isoprenaline (higher efficacy β-AR agonist) in HEK-293 cells and human airway smooth muscle cells respectively. Salmeterol caused less β-AR phosphorylation and internalisation than the full agonist formoterol (January et al., 1997; 1998). Also, salmeterol showed weak efficacy for activation of adenylyl cyclase (Tran et al., 2004), recruitment of arrestin (Moore et al., 2007) and activation of GRK (Tran et al., 2004). Salmeterol did not induce significant β_2-AR internalisation or degradation

in HEK-293 cells and was incapable of stimulating the translocation of arrestin (Moore et al., 2007). Thus, A61603 may be able to induce greater GRK/PKC activity than phenylephrine and this may be responsible for the greater desensitisation by A61603.

In contrast, some reports have shown that efficacy of agonist is not proportional to desensitisation. For example, for β-ARs, Cooper et al., (2011) observed that formoterol and salmeterol-induced similar desensitisation in human lungs. However, Cooper et al., (2011) experiments were based on 12 hours prior incubations with the β-AR agonists, which may reduce the tissue responses, thus, making it impossible to observe the effect of the two β-AR agonists.

Thus, the result presented here is novel. We present results showing that phenylephrine, A61603, but not noradrenaline induced desensitisation to noradrenaline in urethral tissues. Also, desensitisation induced by A61603 was greater than that of phenylephrine. It appears from this study that the α_1-ARs present in the porcine urethra are susceptible to desensitisation when subjected to prolonged stimulation. The data presented in this study also reveals that desensitisation of α_1-AR-mediated responses may be related to agonist specificity and efficacy.

Based on the result that A61603 and phenylephrine induced desensitisation of the subsequent urethral tissues responses to noradrenaline, we analysed the signalling pathways that may be involved. Specific inhibitors of extracellular Ca^{2+} influx, sarcoplasmic reticulum Ca^{2+}release, PKC and Rho kinase were used to reveal the role of Ca^{2+}, PKC and Rho kinase in mediating agonist-induced desensitisation in these tissues.

Do changes in Ca^{2+} mobilisation contribute to desensitisation of α_1-adrenoceptor-mediated responses?

Key to α_{1A}-AR agonist-mediated contraction is an increase in $[Ca^{2+}]i$. A reduction in receptor-mediated Ca^{2+} mobilisation was consistent with α_1-ARs desensitisation, as seen for α_{1B}-ARs (Casas-González et al., 2000; González-Arenas et al., 2006; Molina-Muñoz et al., 2006, 2008). Prior stimulation of α_{1B}-ARs with lysophosphatidic acid reduced intracellular Ca^{2+} induced by noradrenaline in Rat-1 fibroblasts cells (Casas-González et al., 2000). Casas-

González et al., (2000) found that reduction of [Ca^{2+}]i results from depleted Ca^{2+} stores. However, other studies showed that desensitisation was dependent on [Ca^{2+}]$_i$ of the F11 cells (Wu et al., 2013) and neurones (Yao et al., 2010), though, with the 5-HT_{1A} receptor. The desensitisation was blocked by chelation of [Ca^{2+}]i (Wu et al., 2013). Thus, desensitisation of the urethral responses may be dependent on [Ca^{2+}]i. In this section, we examined Ca^{2+} as a modulator of desensitisation in the urethral tissues. Moreover, we elucidated the specific source of Ca^{2+} that may affect the desensitisation to noradrenaline.

The inhibition of extracellular Ca^{2+} influx via the L-type Ca^{2+} channels with nifedipine (1μM) abolished desensitisation mediated by A61603 but not phenylephrine. However, inhibition of intracellular Ca^{2+} release from the sarcoplasmic reticulum did not have any significant effect on the potency of noradrenaline or agonist-induced desensitisation. This suggests Ca^{2+} influx via the L-type Ca^{2+} channels as a contributor to A61603 but not phenylephrine-induced desensitisation. Furthermore, intracellular Ca^{2+} release from the sarcoplasmic reticulum does not contribute to A61603 or phenylephrine-induced desensitisation. In support of this result, although for β-AR, Ikenouchi and colleague, (2008) has reported Ca^{2+} influx via L-type Ca^{2+} channels involvement in desensitisation in airway smooth muscle. In their report, the presence of verapamil, an inhibitor of L-type Ca^{2+} channels, reversed the reduced responsiveness to isoprenaline and forskolin after a prior incubation with platelet-derived growth factor (Ikenouchi et al., 2008). In contrast, Molina-Muñoz et al., (2006; 2008) reported a reduction in [Ca^{2+}]$_i$ for α_{1B}-ARs phosphorylation and desensitisation. However, the Rat-1 fibroblast cells were pre-incubated with insulin-like growth factors. Also, their report was based on prior stimulation of α_{1B}-ARs with noradrenaline. Desensitisation of α_{1B}-ARs was also associated with decreased intracellular inositol phosphates (Molina-Muñoz et al., 2006; 2008). Some reports have shown that [Ca^{2+}]i reduces desensitisation because the Ca^{2+}-calmodulin complex inhibits GRK activity (Chuang et al., 1996; Pronin & Benovic, 1997).

Our results thereby suggested that A61603-induced desensitisation may be mediated by an action on L-type Ca^{2+} channels during agonist exposure. Receptors such as the β_2-AR are closely associated with L-type Ca^{2+} channels in ventricular myocytes (Balijepalli et al., 2006). Moreover, Patriarchi et al., (2016) observed that displacement of the β_2-AR by S1928 phosphorylation from L-type Ca^{2+} channels was a uniquely specific desensitisation

mechanism of the channels in mouse. Based on this report a clear interaction between AR and the L-type Ca^{2+} channels exists. Thus, we show for the first time that A61603-mediated desensitisation of subsequent response to noradrenaline is dependent on L-type Ca^{2+} channels.

Do Rho kinase and PKC contribute to desensitisation of α_1-adrenoceptor-mediated responses?

Evidence indicates that desensitisation of α_1-ARs is mediated through phosphorylation of the carboxyl terminus of the receptor (Diviani et al., 1997; Lattion et al., 1994) by kinase e.g. GRK. However, Rho kinase is associated with myosin light chain phosphatase inhibition and Ca^{2+} sensitization (Teixeira et al., 2007). Thus, we tested the effect of inhibition of Rho kinase on the desensitisation to noradrenaline, after incubation with A61603 or phenylephrine. Inhibition of Rho kinase with fasudil (10μM) eliminated desensitisation of α_1-AR-mediated responses in tissues incubated with either A61603 or phenylephrine. The inference from this result is that Rho kinase contributed greatly to desensitisation of urethral tissues responses to noradrenaline after prior incubation with A61603 and phenylephrine. Fasudil, also known as HA1077, is a cyclic nucleotide-dependent protein kinase and Rho kinase inhibitor (IC_{50} = 10.7μM). It shows good selectivity for Rho kinase over PKC and myosin light chain kinase (Sward et al., 2000; Uehata et al., 1997). However, at concentrations higher than 10μM, fasudil may cause an inhibition of cyclic AMP- and cyclic GMP-dependent protein kinases (Asano et al., 1989). Since in this study we used 10μM fasudil, the result suggests that sustained exposure of the urethra to A61603 or phenylephrine leads to α_1-AR desensitisation and that Rho kinase contributes significantly to this phenomenon.

The modulation of desensitisation by Rho kinase may act as a feedback mechanism for preventing chronic overstimulation of tissues. Thus, a process by which pathways associated with Ca^{2+} sensitization in smooth muscles can also result in receptor desensitisation, probably by phosphorylation when overstimulation of receptors ensues. Cario-Toumaniantz and colleague (2012) suggested a similar model in which Rho kinase had a negative-feedback on its signalling, by downregulating RhoA-guanine nucleotide exchange factor in arteries of rats. The inhibition of Rho kinase activity based on the level of MYPT1 phosphorylation inhibition, prevented agonist-induced down-regulation of the nine RhoA- guanine nucleotide exchange factor transcripts (Cario-Toumaniantz et al., 2012).

Protein kinase C is another kinase associated with α_{1A}-ARs desensitisation (Akinaga et al., 2013; Vázquez-Prado et al., 2000), and agents that activate PKC attenuate responses to agonists (Akinaga et al., 2013; Grandordy et al., 1994). For example Akinaga et al., (2013) showed that α_{1A}-ARs activated by noradrenaline were phosphorylated to a large extent by PKC before being internalised. Thus, we investigated the contribution of PKC to agonist-induced desensitisation by A61603 and phenylephrine.

Inhibition of PKC with calphostin C (1μM) confirmed that PKC contributed to A61603 but not phenylephrine-induced desensitisation. Moreover, inhibition of PKC did not have any significant effect on the agonist potency. Since PKC can also induce phosphorylation of un-activated receptors (Casas-González et al., 2003), A61603 may be mediating a heterologous form of desensitisation. However, phenylephrine appears to induce desensitisation via a PKC-independent mechanism. A previous report by Johnson et al., (2006) reported a similar instance of which closely related agonist-mediated distinct mechanisms of desensitisation in the HEK- 293 cells. They showed that μ opioid receptor agonists morphine and [D-Ala^2, *N*-MePhe^4, Gly-ol^5] enkephalin induced desensitisation by different mechanisms, to a large extent by PKC or GRK-dependent respectively. In the pig urethra, A61603-induced desensitisation involved PKC. The PKC activated by A61603 can also enhance homologous desensitisation (Boterman et al., 2006). Also, PKC can mediate changes in the cellular expression and activity of proteins associated with homologous desensitisation (Winstel et al., 1996).

Possible mechanisms to explain PKC-independent desensitisation by phenylephrine include receptor phosphorylation via GRK or mechanism may be phosphorylation independent. For example, activation of α_1-ARs with phenylephrine resulted in the GRK2-mediated phosphorylation of the β_2-ARs in the human prostate (Hennenberg et al., 2011). On the other hand, Bundey & Nahorski, (2001) reported that activation of α_{1B}-ARs in SH-SY5Y human neuroblastoma cells reduces mAChR-Gαq/11 protein coupling without an increase in mAChR phosphorylation.

Thus desensitisation of urethral α_{1A}-AR occurs and the mechanism involved is agonist-dependent, but appears to include changes in the Ca^{2+}-sensitisation pathway and PKC.

Conclusion

- Tone and contractions of porcine urethra to A61603 are maintained to a greater extent than those to phenylephrine and noradrenaline for intact tissues.
- The urothelium/LP does not contribute to the maintenance of urethral tone induced by A61603.
- Tone maintenance in urethral tissues in response to A61603 does not depend on Rho kinase or prostaglandins.
- A61603 and phenylephrine, but not noradrenaline, induce desensitisation of subsequent noradrenaline responses.
- The mechanisms involved in the process of desensitisation depend on the agonist.
- Rho kinase contributes to phenylephrine-induced desensitisations, while L-type Ca^{2+} channels, Rho kinase and PKC all contribute to A61603-mediated desensitisation (Table 6.10).

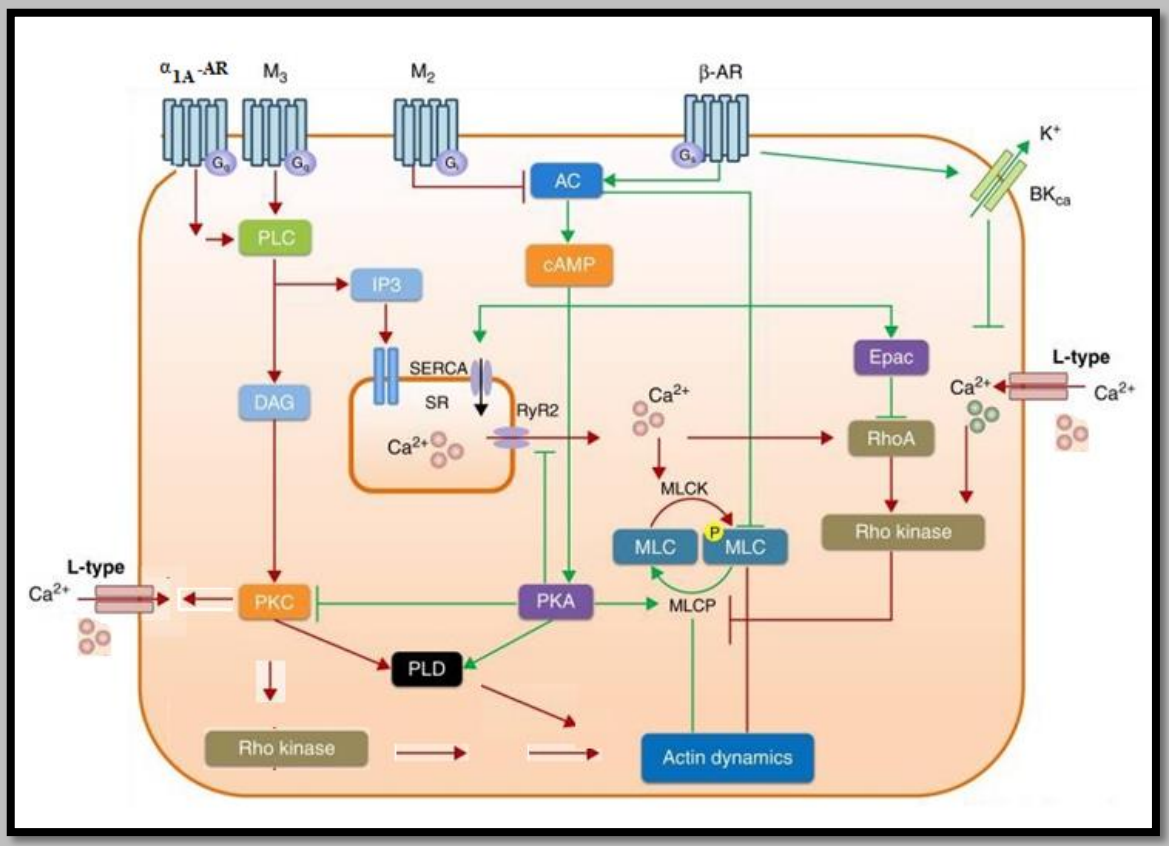
	L-type Ca^{2+} channels	**Ca^{2+} influx via sarcoplasmic reticulum**	**Rho kinase**	**PKC**
Phenylephrine	no	no	yes	no
A61603	yes	no	yes	yes

Table 6.10 The pathways contributing to A61603 and phenylephrine-induced desensitisation of urethral tissues responses to noradrenaline.

Clinical implication

Several α_1-AR agonists, particularly those with selectivity for the α_{1A}-AR subtype, have been developed preclinically for stress urinary incontinence. However, none of these compounds is available for clinical usage. Desensitisation and severe side effect are the main limitations. This study analysed pathways associated with desensitisation of α_1-ARs. Based on our result, agonists with higher potency and efficacy are recommended for maintaining higher urethral luminal pressure. However, these agonists can also induce greater desensitisation of physiological responses. Thus a balance between maximal urethral luminal pressure and desensitisation need to be considered for these agonists.

CHAPTER 7



Modified from Dale et al., 2014.

7 THE POST-JUNCTIONAL INTERACTION BETWEEN ADRENERGIC AND MUSCARINIC PATHWAYS IN THE URETHRA

7.1 RECEPTOR OLIGOMERIZATION

Mutual interaction between GPCR families has been shown and results in distinct functional and pharmacological properties (Ecke et al. 2008; Uberti et al., 2005). Homo- and hetero-oligomerization have been demonstrated for the α_{1A}-AR, α_{1B}-AR and α_{1D}-AR subtypes in recombinant systems (Hague et al., 2004a; Stanasila et al., 2003; Uberti et al., 2005). Hague et al., (2004a) showed α_1-AR heterodimerization in HEK-293 cells which was subtype-specific, with α_{1B}-ARs interacting with α_{1A}- or α_{1D}-ARs. Uberti et al., (2003; 2005) also showed in HEK-293 cells that α_1-AR interactions are subtype-specific. Alpha$_{1B}$-ARs interacted with α_{1A}- or α_{1D}-ARs, but with no detectable interactions occurred between α_{1A}- and α_{1D}-ARs. Moreover, heterodimerization of these receptors did not alter ligand-binding properties but, rather, resulted in increased receptor expression. In particular, α_{1B}/α_{1D}-AR hetero-dimerization increased surface expression of α_{1D}-ARs. Also, confocal imaging confirmed that co-expression of α_{1D}-AR with β_2-AR resulted in translocation of α_{1D}-AR from intracellular sites to the plasma membrane (Uberti et al., 2005). Co-expression of α_{1D}-AR with β_2-AR significantly enhanced the coupling of α_{1D}-AR to noradrenaline-stimulated Ca^{2+} mobilisation. Heterodimerization of AR also confers the ability to co-internalize with other different AR subtype upon agonist stimulation (Stanasila et al., 2003). Thus, heterodimerisation reveals a novel mechanism by which different AR subtypes may regulate each other's activity. Also, heterodimerisation suggests crosstalk between AR, and suggests that receptor interaction may mediate trafficking of the GPCR (Stanasila et al., 2003; Uberti et al., 2005).

Copik et al., (2009) reported that interaction between α_{1A}-ARs and β_2-ARs led to increases in Ca^{2+} influx from the extracellular compartment which was greater than when each receptor was activated. Activation of α_{1A}-ARs and β_2-ARs with noradrenaline, or combined treatment with A61603 and isoprenaline, mediated greater Ca^{2+} influx in HEK-293 cells. However,

little or no increase in intracellular Ca^{2+} release was reported. Thus heterodimerisation also modulates intracellular signalling and Ca^{2+} mobilisation.

Adrenoceptor interactions with other GPCRs has been shown to lead to trafficking of the non-AR (Hague et al., 2004b; Hudson et al., 2010). For example, interactions between β_2-AR and the CB1 cannabinoid receptor (Hudson et al., 2010) or the M71 olfactory receptor (Hague et al., 2004b) lead to trafficking of these GPCRs to the cell surface. Hague et al., (2004b) showed that co-expression of olfactory receptors with β_2-ARs increased olfactory receptors surface expression in HEK-293 cells. A persistent physical interaction between these receptors was demonstrated by co-immunoprecipitation and by co-internalization of the two receptors in response to their specific ligands. Co-expression of β_2-AR and CB1 cannabinoid receptor also altered the signalling properties of CB1 cannabinoid receptor (Hudson et al., 2010). Therefore, dimerization with other non-AR modulates signalling and trafficking of non-ARs.

7.2 INTERACTION BETWEEN AUTONOMIC G-PROTEIN-COUPLED RECEPTORS

The sympathetic and parasympathetic systems are complex and mutually interacting (Dhein et al., 2013; Pera & Penn, 2014). Adrenoceptors and mAChRs exhibit cross regulation in many tissues including the heart (Borst et al., 1997; Dhein et al., 2013), airway smooth muscle (Pera & Penn, 2014) and bladder (Yamanishi et al, 2002a, d). Sympathetic denervation sensitised cardiac responses to catecholamines and concomitantly reduced mAChR expression in the dog heart (Vatner et al., 1985). Also, chronic β_1-AR antagonist treatment desensitised mAChR function in human (Motomura et al., 1990) and rat (Borst et al., 1997; Marquetant et al., 1992) heart. Thus, the AR and mAChR systems seem to interact.

Pre-junctional interaction between G-protein-coupled receptors

Prejunctional auto- and heteroreceptors can modulate transmitter release from parasympathetic and sympathetic nerve endings, with M_2 (and perhaps M_4) mAChR typically inhibiting transmitter release from both types of nerve terminals, and M_1 mAChR and β_2-AR facilitating it in the mouse (Trendelenburg et al., 2005). Studies in the bladder have focused

on mAChR autoreceptors regulating acetylcholine release and have demonstrated a role for facilitatory M_1 mAChRs and inhibitory M_2 and M_4 mAChRs in rat (D'Agostino et al., 1997; Lawrence et al., 2010; Somogyi et al., 1996), mouse (Ehlert et al., 2007), rabbit (Tobin & Sjogren, 1995) and human (D'Agostino et al., 2000) bladder. In the mouse bladder, carbachol reduced evoked noradrenaline release by 57-71% (Trendelenburg et al., 2005). Specifically, M_2 and M_4 mAChRs mediated this inhibition, because noradrenaline release was not recorded in bladders from M_2 and M_4 mAChR knockout animals (Trendelenburg et al., 2005). Thus pre-junctional mAChRs modulate acetylcholine and noradrenaline release.

In other tissues, mAChR activation inhibited the release of endogenous noradrenaline from field stimulated rabbit trachea (Hey et al., 1994). Also, in the horse (Zhang et al., 1995; 1998) and guinea-pig (Belvisi et al., 1996) trachea, β_2-ARs augmented acetylcholine release. Zhang et al., (1998) showed that the β_2-AR agonists, albuterol and formoterol augmented acetylcholine release in a concentration-dependent manner in the horse trachea. In contrast, inhibition of acetylcholine release by β-AR agonists was observed in rat (Wessler et al., 1994) and bovine airways (Brichetto et al., 2003). Thus, the nature of prejunctional interaction of the β-AR on the mAChR system seems to be species related.

In the urethra of human and rabbit Mattiasson and colleagues (1984) have shown a negative feedback regulation of the release of noradrenaline from adrenergic nerve endings. Pre-junctionally located α_2-ARs mediated the feedback. Likewise, an interaction at the nerve terminal level between the adrenergic and cholinergic systems was reported (Mattiasson et al., 1984). Carbachol inhibits release of noradrenaline from adrenergic nerve terminals and acetylcholine from cholinergic nerve terminals, by acting upon pre-junctional mAChRs, thus decreasing urethral tone and intra-urethral pressure (Mattiasson et al., 1984), providing evidence for prejunctional interaction between ARs and mAChRs in the urethra.

Post-junctional interaction between G-protein-coupled receptors

Past reports have demonstrated post-junctional interaction between ARs and non-ARs, which may modulate the signalling of the other (Budd et al., 1999; Miyoshi et al., 2007). For example, insulin-like growth factor-I receptors, sphingosine-1-phosphate receptors, mAChRs, μ-opiod receptors and epidermal growth factor receptors participate in signalling crosstalk

with ARs in cells such as CHO and fibroblasts (Budd et al., 1999; Castillo-Badillo et al., 2012; Dang et al., 2012; Molina-Muñoz et al., 2006; 2008; Vázquez-Prado et al., 1997). The interaction between these receptors and AR modulates signalling of the AR and vice versa. Receptor interaction also modulates sorting and recycling of the ARs. For example, sphingosine-1-phosphate receptors stimulation in HEK-293 cells induced rapid and transient α_{1B}-AR interactions with Rab monomeric G-proteins (a family of Rho guanosine triphosphatases located in endosomes (Seachrist et al., 2000)) thereby targeting and regulating the α_{1B}-AR traffic from late endosomes to lysosomes and recycling to the trans-Golgi (Castillo-Badillo et al., 2015). This indicates that an interaction between sphingosine-1-phosphate receptors and α_{1B}-AR induces heterologous desensitisation of α_{1B}-ARs. Thus, the post-junctional interaction between AR and other non-ARs modulates desensitisation, trafficking, and sorting of the receptors.

Crosstalk has also been observed between AR subtypes. Liang et al., (1998) observed a rapid crosstalk between the α_{2A}-ARs and α_{1B}-ARs co-expressed in CHO cells. Upon selective activation of α_{1B}-ARs with phenylephrine, the function of α_{2A}-ARs was desensitised (Liang et al., 1998).

Post-junctional receptor interaction in the lower urinary tract

Post-junctional interaction has been reported in the lower urinary tract. For example, interaction of mAChRs with β-ARs in the bladder occurs, as shown by studies using pharmacological inhibitors (Witte et al., 2011) and mAChR subtype knock-out mice (Ehlert et al., 2007; Matsui et al., 2003). Witte et al., (2011) showed that the concomitant presence of the mAChR agonist carbachol could attenuate relaxation responses by reducing potency and/or efficacy of β-AR agonists such as isoprenaline. Witte et al., (2011) observed that both M_2 and M_3 -mAChRs contributed to attenuation of β-AR-mediated relaxation of rat urinary bladder, and the M_3-mAChR-mediated effect was via phospholipase C and not PKC. Michel, (2014) also observed that β-AR agonist pre-treatment reduced contractile responses to the mAChR agonist carbachol in rat urinary bladder smooth muscle. Thus, β-AR agonists mediate a functional antagonism of mAChR in the bladder. Roosen et al., (2008) showed a synergistic interaction between the mAChR and α_1-AR system in the trigone of the guinea-pig. Electrical field stimulation-evoked contractions of the guinea-pig trigone were enhanced

by a mAChR agonist and an AR agonist, and the activation of the α_1-AR in the guinea-pig trigone augmented mAChR-mediated responses. In addition, Nagahama et al., (1998) have also shown a postjunctional interaction of the mAChRs with α-ARs in the proximal urethra of the rabbit.

The prototypical primary signalling pathway of M_2 -mAChRs is via inhibition of adenylyl cyclase; whilst M_3 mAChRs and α_{1A}-ARs stimulate phospholipase C, and β-AR signalling is via adenylyl cyclase leading to the formation of cyclic AMP, which can activate protein kinase A (Frazier et al., 2008) (Figure 7.1). Moreover, β-ARs can couple to activation of several potassium channels, mostly large conductance, Ca^{2+}-activated channels as shown in urinary bladder smooth muscle of mouse (Brown et al., 2008; Sprossmann et al., 2009). M_3 mAChR and α_{1A}-AR stimulation leads to the formation of inositol phosphates and diacylglycerol, which in turn mobilise Ca^{2+} from intracellular stores and activate PKC, respectively. Additionally, they can be coupled to a phospholipase D as a downstream event (Wegener et al., 2014), mediate myosin light chain phosphorylation and activation of Rho kinase. Given the role of Ca^{2+} in initiating a smooth muscle contraction, phospholipase C activation is the molecular basis of mAChR and α_{1A}-AR-mediated smooth muscle contraction. An overview of the signal transduction pathways of mAChR and AR in smooth muscle cells showing crosstalk between receptor-mediated signalling is shown in Figure 7.1.

7.3 DESENSITISATION OF MUSCARINIC RECEPTOR

Yoshida et al., (2014) showed that the third intracellular loops of mAChR M_2 and M_4 mediate agonist-dependent internalisation and recycling in HEK-293 tsA201 cells transiently expressing M_2, M_4, and M_2-M_4 chimera mAChRs. Treatment with carbachol (1 mM) for 1 hour reduced numbers of cell surface M_2 and M_4 mAChRs by 40-50% (Ockenga & Tikkanen, 2015). The M_2 and M_4 mAChRs, when chronically activated, mediated inhibition of adenylyl cyclase, intracellular Ca^{2+} concentration ([Ca2+]i) increase, and phospholipase C activation by 60-70% (Krudewig et al., 2000). Thus, mAChRs undergo desensitisation and internalisation usually, in a manner similar to previously described for ARs.

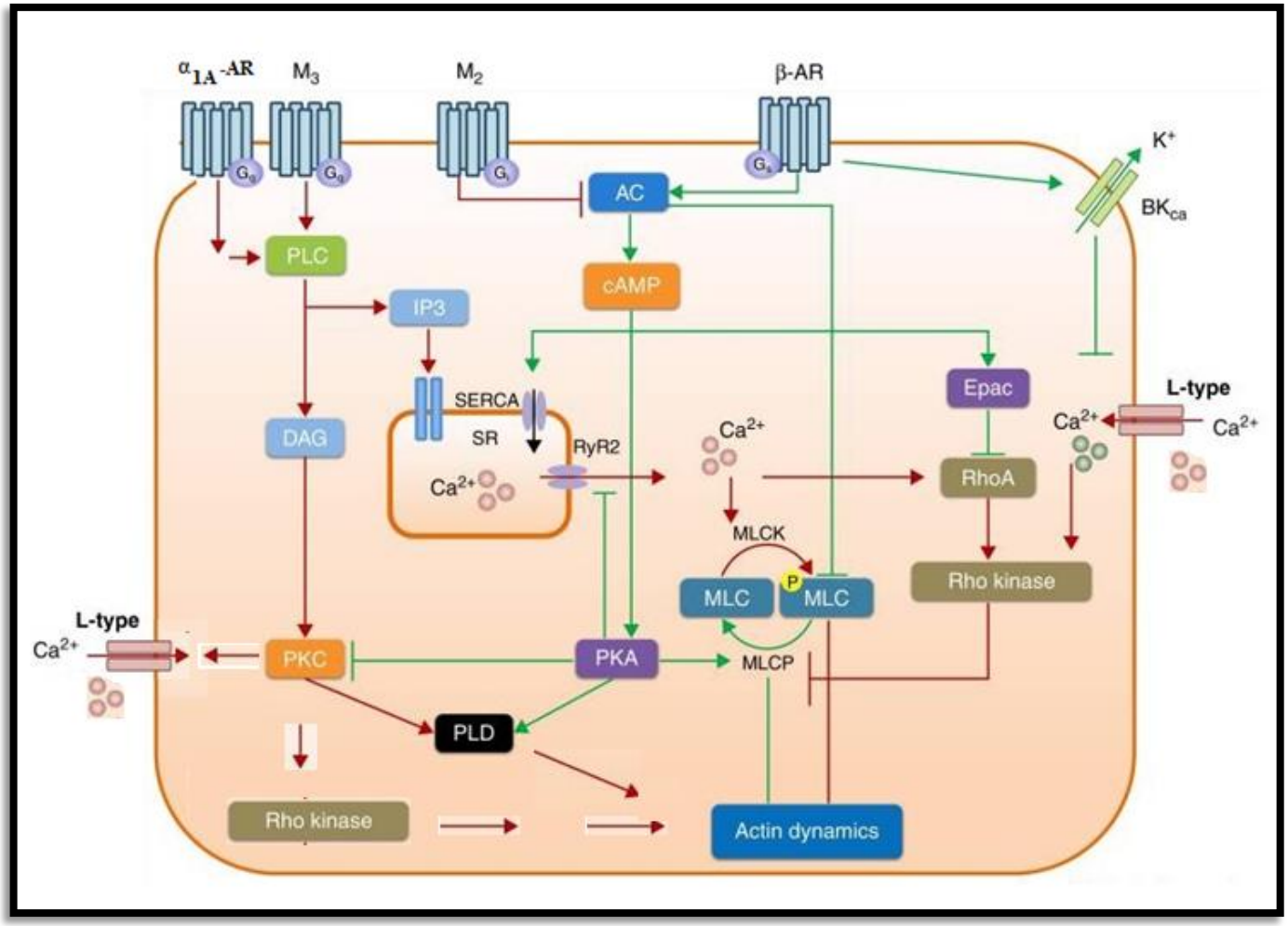


Figure 7.1 Schematic representation of signal transduction pathways involved in the regulation of smooth muscle contraction and possible crosstalk between muscarinic and adrenergic pathways. The α_{1A}-AR, M_2 and M_3 mAChRs mediate contraction, while the β-AR mediates smooth muscle relaxation by activating adenylate cyclase and opening of potassium channels. AC= adenylyl cyclase; AR= adrenoceptor; BK_{Ca}= large conductance Ca^{2+}-activated K^+ channels; DAG= diacylglycerol; Epac = exchange protein directly activated by cyclic AMP; IP3= inositol-tri-phosphate; MLC= myosin light chain; PKA= protein kinase A; PKC= protein kinase C; PLC= phospholipase C; PLD= phospholipase D; RyR= ryanodine receptor; SERCA= sarco/endoplasmic reticulum Ca^{2+}-ATPase; SR= sarcoplasmic reticulum. Red lines and arrows represent pathways activated by muscarinic receptors and α_{1A}-adrenoceptors while green lines and arrows represent pathways activated by β-adrenoceptors. Modified from Dale et al., (2014), Frazier et al., (2008) & Schneider et al., (2004a).

Using radioligand binding techniques, the rank orders for the rate of carbachol-induced internalisation of the mAChR subtypes were $M_2 > M_4 = M_5 > M_3 = M_1$ (Thangaraju & Sawyer, 2011). These observations were based on post-exposure of CHO cells stably expressing mAChR subtypes to carbachol treatment (1 mM). Thangaraju & Sawyer, (2011) further reported that M_1, M_3, M_4 and M_5 mAChRs recycled back to the plasma membrane after 1-hour carbachol treatment. However, the M_2 mAChR was not recycled back to the plasma membrane, suggesting a difference in mAChR desensitisation mechanisms. Moreover, the M_2 and M_4 mAChRs, which are analogous and share several signal transduction pathways show different endocytosis and post-endocytic trafficking routes. After agonist induction, the M_2 mAChR was internalised via a clathrin-independent endocytosis pathway (Delaney et al., 2002). These receptors are then targeted to lysosomes for degradation (Mosser et al., 2008). In contrast, the M_4 mAChR was internalised through the clathrin-dependent endocytosis pathway (Wan et al., 2015) and then recycled back to the plasma membrane. This literature suggests a difference in mechanism of internalisation of mAChR subtypes.

A 5-minute pre-exposure of CHO-M_3 mAChR cells to carbachol resulted in attenuation of the initial peak inositol 1, 4, 5-tris-phosphate response to a subsequent application of the agonist (Tobin et al., 1992). Tsuga et al., (1994) also showed that phosphorylation of M_2 mAChR by GRK2 facilitates sequestration in COS 7 or BHK-21 cells in an agonist-dependent manner. Cha et al., (2006) reported desensitisation of mAChRs of bladder smooth muscle strips isolated from mouse, with bladder smooth muscle strips with circulating anti-M_3 mAChR autoantibodies exhibiting lower carbachol-evoked responses than smooth muscle strips without prior treatment. Moreover, repeated pilocarpine (mAChR agonist) injections of mice for six days caused mAChR desensitisation in the agonist-evoked contractile assay (Cha et al., 2006). Thus, it is clear that desensitisation of mAChR occurs, which may depend on GRK activity. To our knowledge, research into desensitisation of mAChR-mediated responses in the urethra has not been reported.

Epithelial linings have been shown to influence desensitisation of smooth muscle. However, in the lower urinary tract little is known regarding the influence of urothelium on receptor interaction. In the rat bladder, Ferguson et al., (2015) observed that inhibition of urothelial P2X3Rs prevented desensitisation of P2X-mediated detrusor muscle contractions during EFS. Thus, suggesting that the urothelium expressed receptor may modulate receptor-mediated

responses by influencing desensitisation. Based on this data, they proposed a pathway involving P2X3R driven paracrine amplification of ATP released from umbrella cells to increase afferent transmission in the suburothelial sensory plexus and desensitisation of P2X1-mediated purinergic detrusor contractions.

Little is known about the role of the urethral urothelium in receptor desensitisation/interaction. The urethral urothelium is likely to be part of a signalling system that involves projections of the neuroendocrine cells, interstitial cells, and sensory nerve endings (Deckmann et al., 2014; Hashimoto et al., 1999; McCloskey, 2010). Thus this study also looks into the effect of the urothelium on receptor interaction and desensitisation of urethral responses.

Thus the aim of this chapter was to investigate post-junctional interaction between mAChRs and α_1-ARs in modulating responses of the female pig urethra and the role of the urothelium. In particular, this study focused on the following questions:

- Can desensitisation of mAChR- mediated responses be demonstrated in the pig urethra?
- What is the role of urothelium in desensitisation of mAChR- mediated responses?
- Does a post-junctional interaction between the AR and mAChR exist in the pig urethra?
- Does activation of the α-AR or mAChR induce a homologous or heterologous type of desensitisation?

7.4 MATERIALS AND METHODS

Strips of female pig urethra were set up in 8ml organ baths and allowed to equilibrate for 60 minutes as described in chapter 2.

Initial experiments were performed to examine the repeatability of concentration-response curves to carbachol. Second curves to carbachol were depressed, with maximum responses reduced from 102.4±23.0mN to 48.6±12.3mN ($n=9$, $p<0.01$) without a change in pEC_{50} (5.4±0.1 and 5.3±0.1 respectively). For this reason, all experiments in this chapter were performed on pairs of identical tissues with only one concentration-response curve performed on each tissue.

To investigate the effect of α_1-AR stimulation on mAChR-mediated responses, control tissues were incubated with Krebs solution for 15 minutes before cumulative concentration-response curves were constructed to carbachol. Test tissues were incubated with EC_{50} concentrations of noradrenaline (7μM), phenylephrine (4μM) or A61603 (0.03μM) for 15 minutes followed by a 30-minute washout before cumulative concentration-response curves were constructed to carbachol.

To investigate the effect of mAChR stimulation on α_1-AR-mediated responses, control tissues were incubated with Krebs solution for 15 minutes before cumulative concentration-response curves were constructed to noradrenaline. Test tissues were incubated with carbachol (4μM; EC_{50} concentration) for 15 minutes followed by a 30-minute washout before cumulative concentration-response curves were constructed to noradrenaline.

In addition, the experiments were repeated in tissues with or without urothelium. Where specified TTX (1μM) or indomethacin (10μM) was added to the tissues to determine any neuronally mediated effects or prostaglandin-mediated effects respectively, on the receptor-mediated responses.

Data analysis

The receptor-mediated responses were analysed and data expressed as described in chapter 2.

7.5 RESULTS

Effect of incubation with adrenoceptor agonists on urethral tissue responses to carbachol

Maximum contractile responses to carbachol were significantly desensitised by a similar degree after 15 minutes incubation with noradrenaline (7μM), phenylephrine (4μM) or A61603 (0.03μM), as shown in Table 7.1 and Figure 7.2. Furthermore, incubation with noradrenaline, but not phenylephrine or A61603, reduced the pEC_{50} values for carbachol ($p<0.01$) (Table 7.1).

Effect of incubation with carbachol on noradrenaline-induced responses

Surprisingly, maximal contractile responses of urethral tissues to noradrenaline following incubation with carbachol (4μM) were greater than the response of control tissues without carbachol incubation (Figure 7.3), although the difference was not statistically significant (Table 7.2). Furthermore, the pEC_{50} values for noradrenaline were similar for tissues with or without prior incubation with carbachol (Table 7.2).

	Control	+ Noradrenaline (7µM)	Control	+ Phenylephrine (4µM)	Control	+ A61603 (0.03µM)
Max response (mN)	100.0±13.5	**54.1±13.3***	88.6±3.2	**51.6±9.5****	124.0±22.5	**72.3±8.8***
pEC_{50}	5.6±0.1	**5.2±0.1****	5.6±0.1	5.4±0.2	5.5±0.1	5.4±0.1
Change in response (%)	45.9±15.0		41.8±12.2		41.7±18.9	
n	8		5		9	

Table 7.1 Desensitisation of carbachol-induced responses by α_1-adrenoceptor agonists in pig urethra. Mean (±SEM) maximum responses and pEC_{50} values for carbachol, with or without prior 15minutes incubation with EC_{50} concentrations of α_1-adrenoceptor agonists. *p<0.05, **p<0.01 vs. control responses (Student's t-test).

	Control	+Carbachol (4µM)
Max response (mN)	113.8±19.9	153.6±22.5
pEC50	5.2±0.1	5.4±0.1
Change in response (%)	35.1±12.0 (potentiation)	
n	**8**	

Table 7.2 Desensitisation of noradrenaline-induced responses by muscarinic receptor agonist in pig urethra. .Mean (±SEM) maximum responses and pEC_{50} values for noradrenaline, with or without prior 15minutes incubation with EC_{50} concentration carbachol (4µM), in pig urethra.

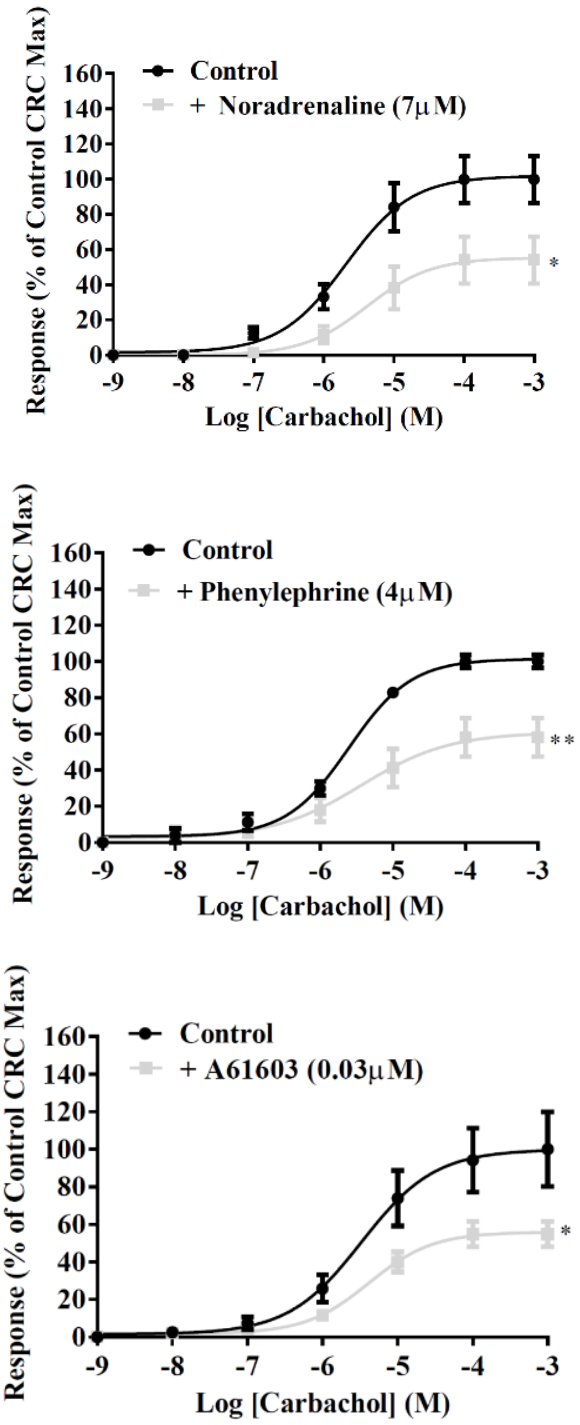


Figure 7.2 Mean (± SEM) concentration-response curves to carbachol in the pig urethral tissues, with or without prior 15 minutes incubation with α-adrenoceptor-agonists, n=5-9. *p<0.05, **p<0.01 vs control responses (Student's t-test).

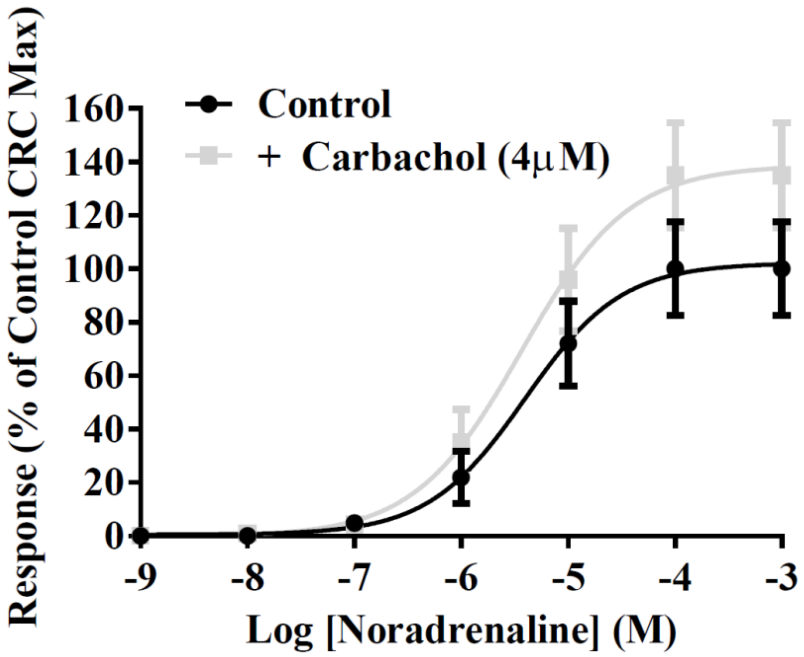


Figure 7.3 Mean (± SEM) concentration-response curves to noradrenaline in pig urethral tissues, with or without prior 15 minutes incubation with carbachol (4μM). n=8.

The influence of the urothelium/lamina propria on adrenoceptor and muscarinic receptor interactions

Maximum responses to carbachol were significantly desensitised to a similar degree, after prior incubation with noradrenaline (7μM) in tissues both with or without urothelium/LP (Table 7.3; Figure 7.4). Furthermore, in the presence of urothelium/LP, the curve for carbachol was shifted leftward significantly by the 15 minutes incubation with noradrenaline (Table 7.3), which was not observed in tissues without urothelium/LP.

Desensitisation of noradrenaline responses after a prior incubation with carbachol was not observed in tissues with intact urothelium/LP, and no change in pEC_{50} values was observed (Figure 7.4). However, maximum responses to noradrenaline, in tissues without urothelium/LP, were desensitised after incubation with carbachol (4μM) for 15 minutes (Figure 7.4C). The pEC_{50} values for noradrenaline following carbachol incubation were also altered, and concentration-response curves were shifted leftward ($p<0.05$) (Table 7.4).

	Responses to carbachol			
	- Urothelium/LP		+ Urothelium/LP	
	Control	+ Noradrenaline (7µM)	Control	+ Noradrenaline (7µM)
Max response (mN)	189.1±22.6	**92.3±19.0****	100.0±13.5	**54.1±13.3***
pEC_{50}	5.8±0.2	5.4±0.1	5.6±0.1	**5.2±0.1****
Percentage difference in Max (%)	51.2±16.2		45.9±15.0	
n	**8**		**8**	

Table 7.3 Alpha$_1$-adrenoceptor desensitisation of muscarinic responses. Mean (±SEM) maximum contractile responses and pEC_{50} values for carbachol before and after prior incubation with noradrenaline. *p<0.05, **p<0.01 vs control responses (Student's t-test).

	Responses to noradrenaline			
	- Urothelium/LP		+ Urothelium/LP	
	Control	+ Carbachol (4µM)	Control	+ Carbachol (4µM)
Max response (mN)	290.0±37.1	**174.9±29.1***	113.8±19.9	153.6±22.5
pEC_{50}	5.7±0.1	**6.1±0.1***	5.2±0.1	5.4±0.1
Percentage difference in Max (%)	39.7±8.5 (depression)		35.1±12.0 (potentiation)	
n	**7**		**8**	

Table 7.4 Muscarinic desensitisation of α_1-adrenoceptor responses. Mean (±SEM) maximum contractile responses and pEC_{50} values for noradrenaline before and after prior incubation with carbachol. *p<0.05 vs control responses (Student's t-test).

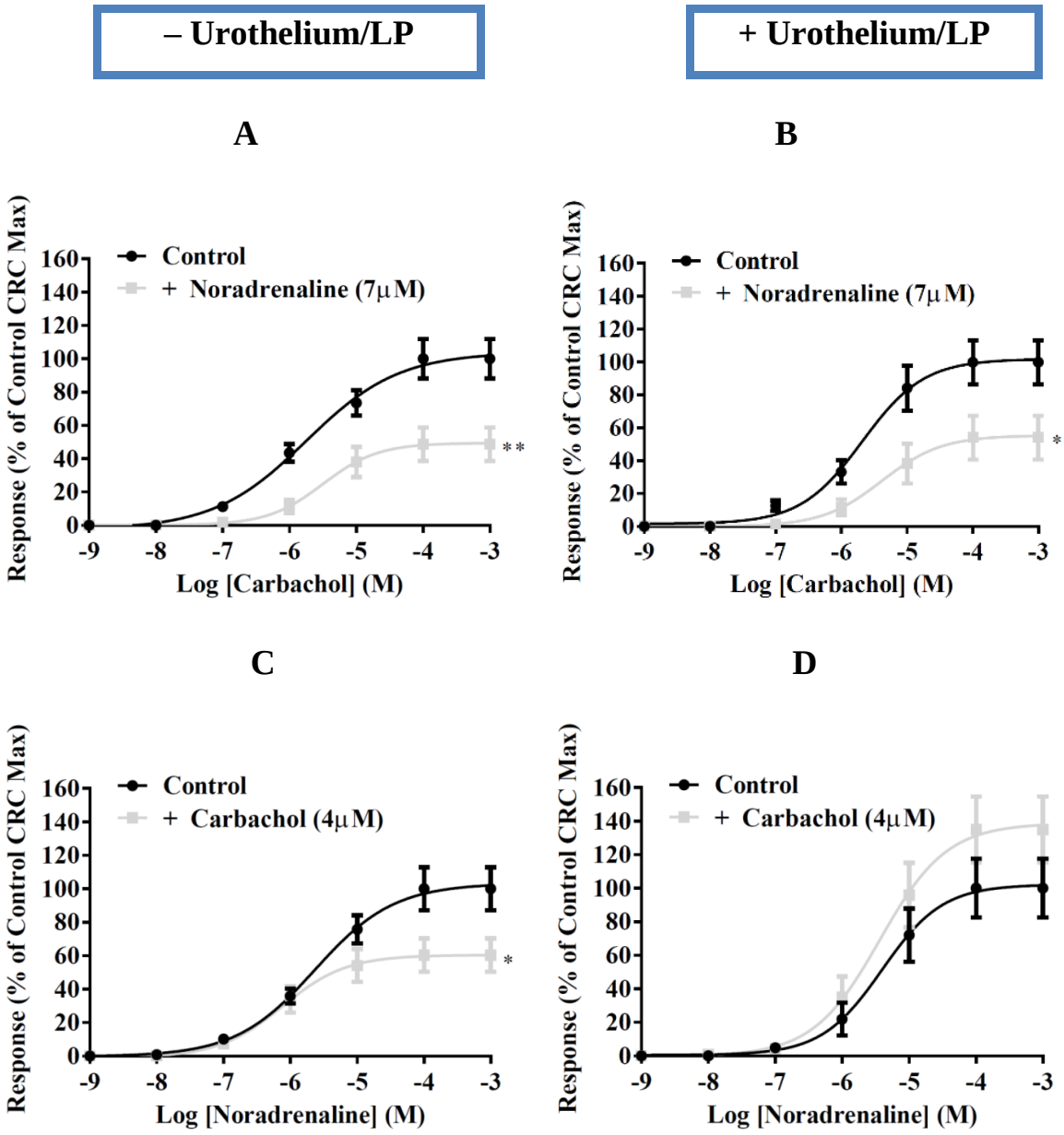


Figure 7.4 Cross desensitisation of muscarinic and α_1-adrenoceptor-mediated responses. Mean (±SEM) concentration-response curves to carbachol (A-B) or noradrenaline (C-D) with or without prior incubation with noradrenaline (7μM) or carbachol (4μM) respectively, in urethral tissues with or without urothelium/LP. n=7-8; *p<0.05, **p<0.01 vs. control response (Student's t-test). LP= lamina propria.

Urethral tissues with urothelium/LP were also incubated with carbachol in the presence of TTX (1μM) to block the neuronal release of neurotransmitter, or indomethacin (10μM) to inhibit cyclooxygenase enzyme. TTX (1μM; Table 7.5) or indomethacin (10μM; Table 7.6) did not have any significant effect on responses to noradrenaline after prior incubation with carbachol (4μM) (Figure 7.5), and the pEC_{50} values for noradrenaline were similar for tissues with or without TTX (1μM) or indomethacin (10μM).

	Responses to noradrenaline			
	-TTX(1µM)		+TTX(1µM)	
	Control	+ Carbachol (4µM)	Control	+ Carbachol (4µM)
Max response (mN)	102.3±11.7	141.7±14.2	260.3±43.0	300.9±58.5
pEC_{50}	5.4±0.3	5.4±0.3	5.4±0.2	5.4±0.2
Percentage difference in Max (%)	37.9±13.4		15.6±21.0	
n	7		7	

Table 7.5 Effect of tetrodotoxin (1µM) on the carbachol (4µM) desensitisation of urethral α_1-adrenoceptor responses. Mean (± SEM) maximum contractile responses and pEC_{50} values for noradrenaline with or without prior incubation with carbachol (4µM).

	Responses to noradrenaline			
	-Indomethacin(10µM)		+indomethacin(10µM)	
	Control	+ Carbachol (4µM)	Control	+ Carbachol (4µM)
Max response (mN)	105.3±11.0	135.4±14.2	294.0±50.2	343.9±68.7
pEC_{50}	5.4±0.3	5.5±0.3	5.4±0.2	5.7±0.1
Percentage difference in Max (%)	27.6±11.2		17.0±26.9	
n	7		8	

Table 7.6 Effect of indomethacin (10µM) on the carbachol (4µM) desensitisation of urethral α_1-adrenoceptor responses. Mean (± SEM) maximum contractile responses and pEC_{50} values for noradrenaline with or without prior incubation with carbachol (4µM).

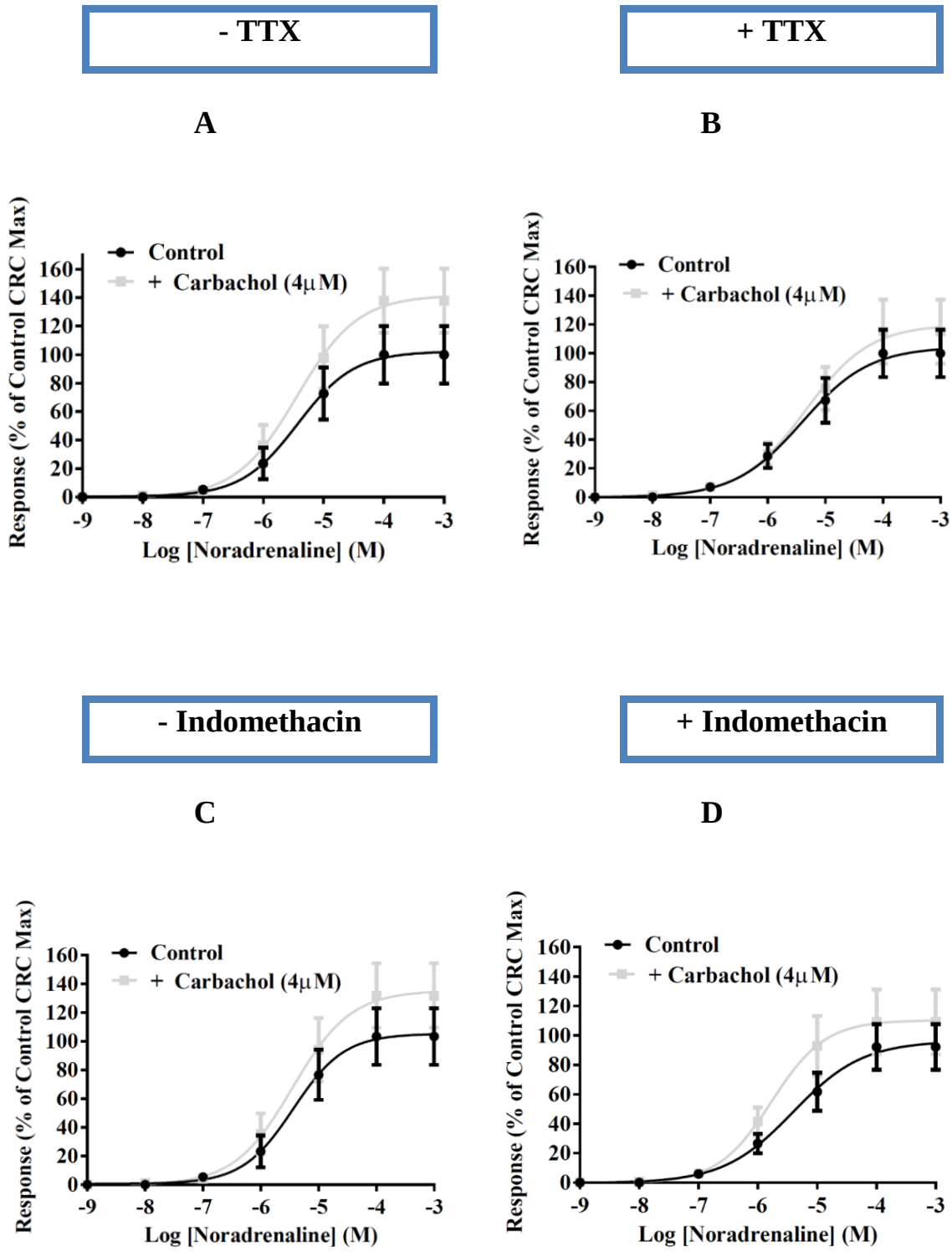


Figure 7.5 Mean ± (SEM) concentration-response curves to noradrenaline with or without prior incubation with carbachol. Experiments were carried out in the presence or absence of TTX (1µM) or indomethacin (10µM). n=7-8.

7.6 DISCUSSION

The interaction between GPCR may modulate tissue responses either by decreasing or potentiating responses (Budd et al., 1999; Bundey & Nahorski, 2001; Charles et al., 2003; Hague et al., 2004a; Stanasila et al., 2003). Interaction between ARs (Hague et al., 2004a; Stanasila et al., 2003; Uberti et al., 2005) and mAChRs (Borst et al., 1997; Dhein et al., 2013; Motomura et al., 1990; Pera & Penn, 2014) is well documented in numerous cells and tissues, although not in the urethra. Thus, we investigated potential interaction of ARs and mAChRs in pig urethral tissues.

Can desensitisation of muscarinic receptor-mediated responses be demonstrated in the pig urethra?

It is also known that the mAChR desensitises, as shown in COS 7 and BHK-21 cells (Tsuga et al., 1994; 1998). Cha et al., (2006) also observed desensitisation of mAChRs in bladder smooth muscle strips isolated from mouse with circulating anti-M_3 mAChR autoantibodies. However, to our knowledge, no work has been published in urethral tissues with regards to mAChR desensitisation. In this study, we observed desensitisation of mAChR-mediated responses in the porcine urethra following prior cumulative concentration-response curves to carbachol (page 236). In addition to carbachol reducing subsequent mAChR-mediated responses, agonist potency was also reduced significantly.

Previous reports have shown that pre-stimulation of CHO cells expressing the human M_1-mAChR with agonist led to desensitisation of inositol 1, 4, 5-tris-phosphate accumulation with a 4-fold shift in the agonist dose-response curve (Waugh et al., 1999). Desensitisation has also been associated with a major reduction in the maximal agonist-induced responses and inositol monophosphate, which was associated with activation and translocation of PKC (Dillon-Carter & Chuang, 1989). Furthermore, desensitisation by carbachol has been shown in primary cultures of corticostriatal neurones and cultured cerebellar granule cells, and it was reported that incubation with carbachol attenuated phospholipase C responsiveness and decreased the number of cell surface mAChRs in these cells (Dillon-Carter & Chuang, 1989). Thus carbachol-induced desensitisation observation in this study may be associated with reduced inositol monophosphate level and PKC activity. The desensitisation may also include

uncoupling of receptors by kinases within seconds after the addition of carbachol, sequestration and or down-regulation of the mAChR as shown in CHO cells by Waugh et al., (1999). Down-regulation of receptors is a possibility, given that downregulation of mAChR has been reported at early time periods in murine fibroblast cell lines (Wei et al., 1994). Also, 1-hour exposure of Fischer rat thyroid-epithelial cells to carbachol resulted in a desensitisation of mAChR-mediated intracellular Ca^{2+} mobilisation and induced the internalisation of constitutively expressed mAChR in this cell type (Montiel et al., 2004). Whether these diminished mAChR-mediated responses are a result of desensitisation or downregulation requires confirmation by further investigations.

Does activation of urethral α_1-adrenoceptors induce desensitisation of muscarinic receptor-mediated responses and vice versa?

In the previous chapter we showed that A61603 and phenylephrine induced desensitisation of α_1-AR-mediated responses. In this section, we investigated the possibility of α-AR agonists inducing desensitisation of mAChR-mediated responses and vice versa in the pig urethra, representing a postjunctional interaction between the AR and the mAChR systems. Post-junctional interaction between the AR and the mAChR systems is well documented, although these studies examined β-AR and other tissues apart from the urethra. For example, Witte et al., (2011) showed that the concomitant presence of a mAChR agonist, can attenuate relaxation responses by reducing potency and/or efficacy of β-AR agonists such as isoprenaline in the rat bladder. They observed that both M_2 and M_3 mAChRs contributed to attenuation of β-AR-mediated relaxation of rat urinary bladder, and the M_3 mAChR-mediated-effects was via phospholipase C but not via PKC. In addition, Budd et al., (1999) showed that activation of M_3-mAChRs mediated heterologous phosphorylation of β_2-ARs in a GRK-independent fashion, via PKC. Heterologous β_2-AR phosphorylation correlated with receptor desensitisation and the reduction in maximal cyclic AMP accumulation.

In this study, a prior incubation (15 minutes) of urethral tissues with noradrenaline, phenylephrine or A61603, at EC_{50} concentrations, induced a significant desensitisation of subsequent mAChR-mediated responses. Also, the potency of carbachol was reduced after 15 minutes incubation with noradrenaline. The desensitisation induced by all three agonists was similar. This result confirms a heterologous type of desensitisation by phenylephrine, A61603

and noradrenaline between AR and mAChR. Past studies on α_1-ARs have revealed an α_1-AR-mediated heterologous desensitisation of mAChR-mediated responses in SH-SY5Y human neuroblastoma cells using noradrenaline (Bundey & Nahorski, 2001). Although these authors showed that α_{1B}-AR stimulation produced a heterologous desensitisation of mAChR-mediated $G_{q/11}$ activation, they could not detect an increase in mAChR phosphorylation.

In preceding chapters, we showed that the potency and efficacy of the α-AR agonist used affected the degree of desensitisation of α_1-AR-mediated responses. This phenomenon suggests that increasing agonist-coupling efficiency primarily affects desensitisation. This may be by increasing the rate of phosphorylation of the ARs (January et al., 1997). However, this phenomenon seems not to apply to the heterologous desensitisation of mAChR by α_1-AR stimulation, since noradrenaline, phenylephrine and A61603 induced a similar degree of heterologous desensitisation of mAChR-mediated responses, suggesting that the three agonists may be equi-effective in mediating heterologous desensitisation of mAChRs. The ability of the α-AR agonists to induce similar heterologous desensitisation may be associated with similar ability to activate second messenger-dependent kinases such as protein kinase A and PKC (Penn et al., 1998). In an earlier chapter we showed that A61603-induced contraction depends on PKC. In contrast, Roosen et al., (2008) showed a synergistic interaction between the mAChR and α_1-AR system in the bladder. Electrical field stimulation contractions of the guinea-pig trigone were enhanced by a mAChR agonist and an AR agonist, and activation of the α_1-AR in the guinea-pig trigone augmented mAChR-mediated responses. This highlights tissue-specific differences in regions of the lower urinary tract.

Upon investigating whether mAChR stimulation caused desensitisation of α_1-AR-mediated responses we found that, prior incubation with carbachol did not desensitise urethral responses to noradrenaline. Instead, a non-significant potentiation was observed. An earlier report by Nagahama et al., (1998) has shown a similar report in the proximal urethra of the rabbit. Carbachol dose-dependently enhanced contraction of the proximal urethra region to noradrenaline. The potentiation may be the result of upregulation of α_1-AR or its signalling in response to mAChR activation. For example, studies, in cardiac tissue (Benes et al., 2012; Dhein et al., 2013) and with cloned receptors in transfected cells (Budd et al., 1999; Lee and Fraser, 1993), showed that desensitization/down-regulation of mAChR or β-ARs can lead to sensitization/up-regulation of co-expressed receptors. Thus, in the present study activation or

desensitisation of the mAChR-mediated responses by carbachol may upregulate the noradrenaline-mediated responses in the urethral tissues by a similar mechanism. In the guinea pig trigone, Ca^{2+} sensitization was responsible for a synergistic potentiating effect between AR, mAChR, and purinergic receptor activation in the muscle of the superficial trigone (Roosen et al., 2008) mediated via PKC and Rho kinase. Thus, activation of PKC and Rho kinase may be responsible for the effect in this study. Either way, carbachol caused desensitisation of subsequent mAChR-mediated responses but not of subsequent α_1-AR mediated responses, confirming a homologous form of desensitisation in the urethral smooth muscle.

Does the urothelium/lamina propria affect the receptor-mediated desensitisation?

In earlier chapters, we showed the inhibitory effect of the urothelium/LP on the underlying urethral smooth muscle responses, and showed that this effect was not associated with prostaglandins or NO. In this chapter, we investigated whether the urothelium/LP influences receptor interaction in the urethra.

The presence of urothelium/LP did not affect the noradrenaline-induced desensitisation of mAChR-mediated responses. However, whilst carbachol was not able to desensitise α_1-AR-mediated responses in urethral tissues with urothelium/LP, it did desensitise α_1-AR-mediated responses in those without urothelium/LP. These results infer a post-junctional interaction dependent on the urothelium/LP. Templeman et al., (2002) observed that urothelium derived factors mediated crosstalk among mAChR and AR systems in the porcine bladder. However, Templeman et al., (2002) reported inhibition of noradrenaline-induced responses in the trigone of the porcine bladder with intact urothelium in the presence of carbachol, an observation we did not see in the urethra. The variation in results may be due to different lower urinary tract tissues.

In the present study, we hypothesised that in the urethra carbachol might stimulate release of a factor from the urothelium that prevents or opposes desensitisation of noradrenaline responses. It is known that the urothelium releases factors in response to receptor stimulation (McLatchie et al., 2014; Sui et al., 2014). Stimulation of mAChRs increases ATP production,

and antagonising these receptors reduces ATP release in the bladder urothelial cells (McLatchie et al., 2014; Sui et al., 2014). Likewise, acetylcholine is released in response to mechanical stress (McLatchie et al., 2014; McLatchie & Fry, 2015). Less is known about urothelially released ATP and acetylcholine and other factors in the urethra.

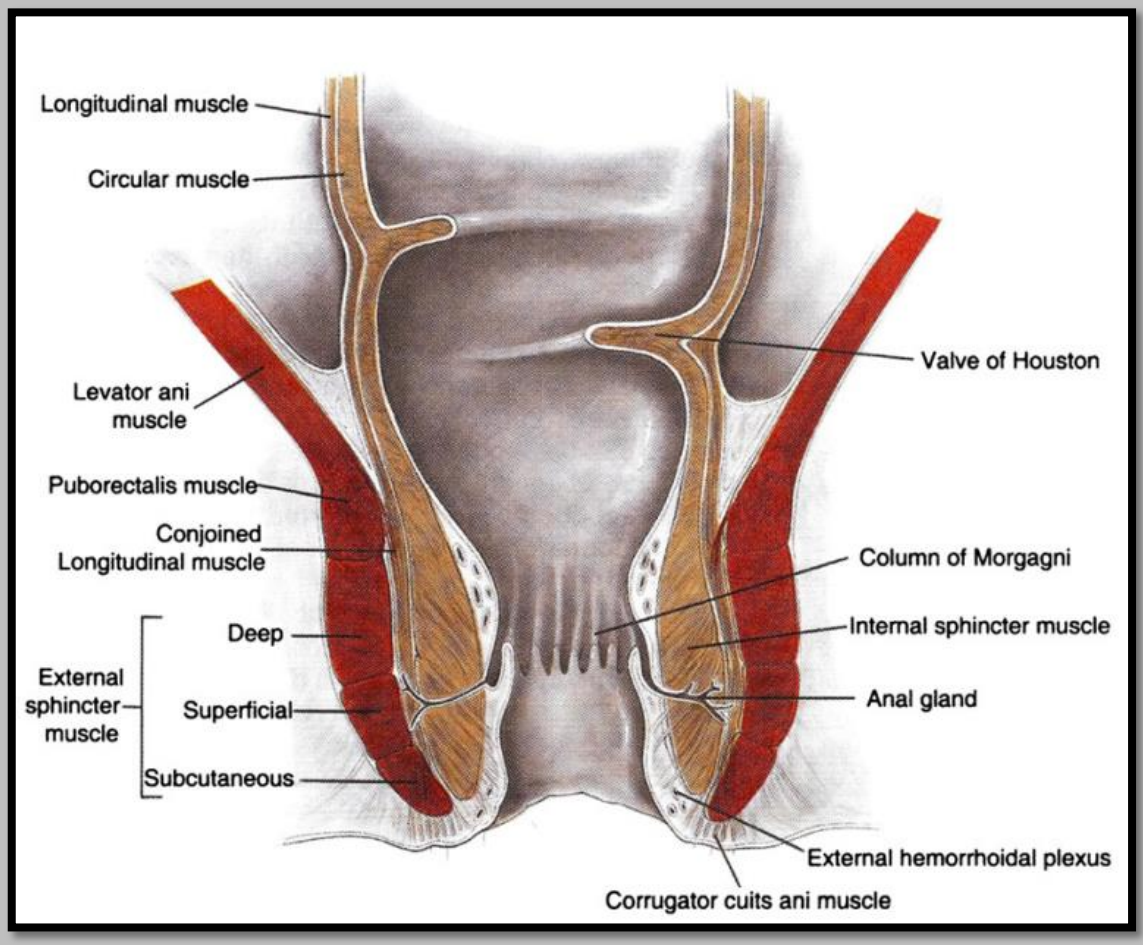
Prospective excitatory factors that may be released from the urothelium/LP include prostaglandins. Nile & Gillespie, (2012) have reported that cholinergic stimulation induces a concentration-dependent production of prostaglandin E_2 in guinea pig bladder urothelium, which was inhibited in a dose-dependent manner by the selective M_2 -mAChR antagonist. To our knowledge, no study has shown release of prostaglandins in the urothelium of urethra. However, expression of the prostaglandin E receptors, EP_1, EP_2, EP_3, and EP_4, and mRNA for EP_2, EP_3, and EP_4 were reported in dog urethral urothelium (Ponglowhapan et al., 2010). In the human urethra, prostaglandin $F_{2\alpha}$ mediates contraction (Andersson et al., 1977) as well as in rabbit urethral tissues (Morita et al., 1994). Thus prostaglandins are a possible modulator opposing desensitisation of the urethral tissues responses to noradrenaline. However, inhibition of cyclooxygenase with indomethacin (10μM) did not result in a desensitisation of noradrenaline-mediated responses ruling out prostaglandins. In addition, prior incubation with TTX (1μM) did not result in a desensitisation of noradrenaline-mediated responses. This suggests that the neuronal release does not play a role.

Thus although the urothelium opposes desensitisation, the precise mechanism remain to be elucidated. This study demonstrates post-junctional interaction between ARs and mAChRs in the urethra.

Conclusions

- Noradrenaline, phenylephrine and A61603 caused desensitisation of carbachol responses in urethral tissues. Desensitisation observed was similar for all agonists.
- Desensitisation induced by noradrenaline was not urothelium/LP dependent.
- Carbachol caused desensitisation of noradrenaline-mediated responses in the urethral tissues, although only in the absence of the urothelium/LP.
- Masking of receptor-mediated desensitisation was not of neuronal origin and not due to cyclooxygenase activity.

CHAPTER 8



Modified from Gordon & Nivatvongs, 2007.

8 NEUROTRANSMITTERS REGULATING CONTROL OF THE INTERNAL ANAL SPHINCTER SMOOTH MUSCLE

8.1 BASAL TONE OF THE INTERNAL ANAL SPHINCTER

The internal anal sphincter smooth muscle, which is a ring-shaped structure encircling the anorectum, exhibits basal tone and controls the passage of faecal contents through the anorectum (Zhang et al., 2016b). Healthy sphincters open transiently during faecal passage but, in the basal state, remain closed and therefore require perpetual force generation from the smooth muscle cells. The loss of this tone leads to disorders such as faecal incontinence, whilst the hypertonic IAS smooth muscle inhibits healing of anal fissure (Farouk et al., 1994). A significant number of studies have indicated that the basal tone of IAS smooth muscle is independent of extrinsic nerve and hormone stimulation, but instead is an intrinsic property of the sphincter smooth muscle itself (Rattan, 2005). Zhang et al., (2016b) showed that intracellular Ca^{2+} is elevated via ryanodine receptors, L-Type Ca^{2+} channels and Ca^{2+}-activated Cl^- channels to mediate IAS basal tone in the mouse. The increase in intracellular Ca^{2+}activates myosin light chain kinase and triggers the basal contractile tone. The Ca^{2+} release from the opening of ryanodine receptors in the sarcoplasmic reticulum of the IAS is the initial signal for the generation and maintenance of IAS smooth muscle basal tone and this increased Ca^{2+} then activates Ca^{2+}-activated Cl^- channels which activated the L-Type Ca^{2+} channels (Zhang et al., 2016b). Also, extracellular Ca^{2+} has been shown to contribute to the maintenance of basal tone of IAS in other animals (Cook et al., 1999a; Mundey et al., 2000) and human (Cook et al., 1999b). Specifically, the Ca^{2+} influx via the L-type Ca^{2+} channels contributes to the basal tone of IAS (Cook et al., 1999a, b).

Rho kinase is also important in the maintenance of IAS smooth muscle basal tone in animals (Patel & Rattan, 2006; 2007; Rattan et al., 2006) and human (Rattan & Singh, 2012; Rattan et al., 2015). Patel & Rattan, (2006) examined the molecular basis for the basal myogenic tone in the IAS and compared it to rectal smooth muscle (a mixture of phasic and tonic), and anococcygeus smooth muscle (phasic muscle) in the rat. The levels of RhoA/Rho kinase were higher at the cell membrane in the IAS compared with those from the rectal smooth muscle

and anococcygeus smooth muscle and C3 exoenzyme (RhoA inhibitor) and Y27632 (Rho kinase inhibitor) caused concentration-dependent relaxations of the IAS. In addition, active Rho kinase-II was shown to cause further shortening of the IAS. Singh et al., (2014; 2015) have shown that an age-associated decrease in IAS tone is due to oxidative stress-mediated decreases in Rho kinase signal transduction in the rat. The decrease in tone was associated with a decrease in RhoA/Rho kinase expression at the transcriptional and translational levels (Singh et al., 2014). Singh et al., (2014) thereby concluded that an increase in oxidative stress plays a major role in the ageing-associated decrease in IAS tone in the aged and may be one of the underlying mechanisms of recto-anal incontinence in certain ageing patients. Furthermore, Singh et al., (2015) showed that reactive oxygen species produced a bimodal effect on IAS smooth muscle tone of the rat. Higher concentrations of reactive oxygen species produced a decline in IAS tone, while lower concentrations of reactive oxygen species induced an increase in RhoA/Rho kinase activity and IAS tone. All these results suggest a contribution by RhoA/Rho kinase to basal tone of the IAS smooth muscle and that altered activity is seen during ageing, leading to anorectum pathological conditions such as faecal incontinence.

The rectoanal region is the final site for controlling the storage, transport and evacuation of faecal materials. The IAS is usually closed and maintains continence, while during the defecation reflex it briefly relaxes to allow the passage of faecal materials. The activity of the IAS is modulated by the innervation of the smooth muscle which is elucidated below.

8.2 INNERVATION OF THE INTERNAL ANAL SPHINCTER

As in other parts of the gastrointestinal tract, contraction and relaxation of the IAS smooth muscle are regulated by enteric and autonomic neurones and by the actions of other cell types, including ICC and fibroblast-like cells (Cobine et al., 2011).

Excitatory motor innervation to the IAS is almost exclusively sympathetic, since it is blocked by guanethidine (inhibits the release of noradrenaline) and is inhibited by α_1-AR antagonists (Cobine et al., 2007; 2010; Glavind et al., 1997; Tichenor et al., 2002). Guanethidine treatment leads to almost complete blockade of the EFS-induced response in the IAS of dog (Tichenor et al., 2002). Also, both prazosin (α_1-AR antagonist) and yohimbine (α_2-AR

antagonist) significantly reduce responses to EFS, but prazosin produces a significantly greater inhibition than yohimbine (Tichenor et al., 2002). However, in the proximal rectum, excitatory motor innervation is mediated by both cholinergic and tachykinergic mechanisms (Tichenor et al., 2002). Thus, extrinsic sympathetic nerves play an important role in regulating sphincter function.

Neurotransmitters, such as NO, play a major role in the inhibitory neurotransmission of the IAS (Rattan et al., 1992; Rattan & Chakder, 1992). However, inhibitory neurotransmission in the IAS may involve other neurotransmitter such as vasoactive intestinal polypeptide (VIP) (Keef et al., 2013; Raghavan et al., 2010; 2011; 2014), carbon monoxide (CO) (Rattan & Chakder, 1993; Watkins et al., 2004) and purines (Acheson et al., 2009; Duffy et al., 2012).

Excitatory inputs

Initial in vitro studies by Parks et al., (1969) demonstrated that the sympathetic neurotransmitter noradrenaline causes a contraction of the IAS smooth muscle that could be blocked by the addition of the α-AR antagonist phenoxybenzamine. Yamato & Rattan (1990) demonstrated that stimulation of α_1-AR causes an increase in resting pressure developed by the sphincter without affecting the anal pressure response to rectal distension. Alpha-AR-mediated responses have been shown in the IAS smooth muscle of animals (Mills et al., 2008; Rayment et al., 2010) and humans (Owaki et al., 2015). In addition, Speakman et al., (1990; 1993) showed that in vitro contractile responses of the human IAS to noradrenaline are decreased in incontinence. Using EFS, Cobine et al., (2007) showed that the excitatory motor innervation to the IAS of the monkey was noradrenergic, and the frequency-dependent contractions were abolished by guanethidine. In the sheep IAS, endogenous noradrenaline acts via postjunctional α-AR to antagonise neurogenic relaxations that are largely mediated by NO (Acheson et al., 2009). Thus, noradrenaline acting via the α-AR is excitatory in the IAS (Table 8.1).

Table 8.1 Neurohumoral substances and their effects in the internal anal sphincter.

Neurohumoral substance	Effects	Species	References
α-AR agonists	Contraction.	Pig, sheep & human.	Acheson et al., 2009; Mills et al., 2008; Jones et al., 2003b; Parks et al., 1969 ; Ramalingam et al., 2010; Rayment et al., 2010; Simpson et al., 2014; Speakman et al., 1990; 1993; Owaki et al., 2015.
NO, and NO donors	Relaxation.	Rat, mouse, opossum & pig.	Folasire et al., 2016.; Rattan et al., 2004; 2005; Rattan & Chakder, 1992; 2000.
CO and CO-releasing molecule (CORM-1)	Relaxation.	Rat, opossum & mouse.	Rattan et al., 2004; 2005; Rattan & Chakder, 1993; Watkins et al., 2004.
Vasoactive intestinal polypeptide (VIP)	Relaxation.	Human, opossum & mouse.	Burleigh et al., 1979; Keef et al., 2013; Raghavan et al., 2011; Rattan et al., 2005; Rattan & Chakder, 2000; Tottrup et al., 1992.
ATP	Relaxation.	Rat, sheep & mouse.	Acheson et al., 2009; De Luca et al., 1999; Duffy et al., 2012; Keef et al., 2013; Opazo et al., 2011; McDonnell et al., 2008.
	Contraction.	Pig.	Folasire et al., 2016.
H_2S and donor	Relaxation.	Mouse & pig.	Dhaese et al., 2010; Folasire et al., 2016.
Muscarinic agonists (ACh, bethanechol, carbachol)	Contraction.	Opossum, mouse, vervet monkey, human.	Duffy et al., 2012; Fan et al., 2001; Park et al., 2013; Rayner, 1979; Somara et al., 2009.
	Relaxation	Pig, cat & human.	Buntzen et al., 1996; Burleigh et al., 1979; Opazo et al., 2009; Ramalingam et al., 2010; Speakman et al., 1992.

Inhibitory inputs

In terms of the inhibitory control of the IAS smooth muscle studies have suggested that nNOS and eNOS isoforms of NOS modulate distinct components of IAS function (Banwait & Rattan, 2003; Evers, et al., 2013). Neuronal NOS was observed in the myenteric plexus of canine gastrointestinal neurones using NADPH-diaphorase staining techniques (Ward et al., 1992), and western blot studies revealed the presence of eNOS in the circular smooth muscle of the IAS of the opossum (Banwait & Rattan, 2003). Likewise, immunoreactivity for nNOS has been shown in the IAS of other animals (Cobine et al., 2011; Wang et al., 2013), and humans (Moszkowicz et al., 2012) and inhibition of nNOS by reactive oxygen species may contribute to pathological conditions of the IAS (Singh et al., 2015). Qu et al., (2008) showed that 90% of NOS-expressing neurones were inhibitory motor neurones to the IAS muscle (26% of all neurones) and 10% (3% of all neurones) were interneurons. Thus, it is clear that NO is the major non-adrenergic, non-cholinergic neurotransmitter mediating neurogenic relaxation of the IAS in animals (Jones et al., 2003a; Rae & Muir, 1996) and human (O'Kelly et al., 1993b).

There is substantial evidence that ICC participate in nitrergic neurotransmission in the gastrointestinal tract. Interstitial cells of Cajal make very close contacts with varicose terminals of enteric motor neurones (Daniel & Posey-Daniel, 1984), express guanylate cyclase (Lino et al., 2009), synthesise cyclic GMP (Shuttleworth et al., 1993), and generate hyperpolarization (Burns et al., 1996) in response to nitrergic stimulation. Imatinib, an inhibitor of Kit receptor tyrosine kinase (a marker for ICC), significantly reduced the tone and the spontaneous activity of the human IAS, which also stained positive for the c-kit protein. Interstitial cells of Cajal have been observed in the IAS of animals (Cobine et al., 2010; 2011; 2014; Duffy et al., 2012) and humans (Lorenzi et al., 2014; Piotrowska et al., 2003), and thus ICC may participate in nitrergic transmission in the IAS.

The cascade underlying nitrergic neuromuscular transmission in the IAS comprises Ca^{2+}-activated NOS, as a generator of NO, and NO-sensitive guanylyl cyclase, as a generator of the second messenger cyclic GMP (Lies et al., 2014). An important receptor for NO in visceral muscle is soluble guanylate cyclase, which is a heterodimeric enzyme containing α and β subunits that convert GTP to cyclic GMP (Denninger & Marletta, 1999). Guanylate

cyclase has been shown to mediate the NO-mediated relaxation of IAS in the mouse (Cobine et al., 2014). The relay of the cyclic GMP signal in the gastrointestinal tract may involve several effector molecules such as cyclic GMP-dependent protein kinase, cyclic GMP-regulated phosphodiesterases, and cyclic GMP-gated ion channels (Figure 8.1; Hofmann, 2005).

Moreover, NO mediates its effect via an important downstream mediator of cyclic GMP, cyclic GMP-dependent protein kinase I (cGKI), which is a serine/threonine kinase that exists in two isoforms, cGKIα and cGKIβ. Cyclic GKI has been shown to phosphorylate ion channels and other proteins responsible for regulating the contractile state of smooth muscles (Orstavik et al., 1997). The cGKI-independent pathway is also implicated in NO-mediated IAS relaxation as shown in the cGKI knockout mouse (Cobine et al., 2014).

Clinically the inhibitory pathways of IAS may be a target for pharmacotherapy i.e. relaxation of the IAS for anal fissures or stop relaxation for faecal incontinence. A deficiency in NO may be associated with chronic anal fissure, since this condition is associated with hypertonia of the IAS which lower the local blood flow and prevents healing (Lund, 2006). Internal anal sphincter from patients with chronic anal fissure showed little NOS presence compared with controls (Lund, 2006).

Adenosine triphosphate (ATP)

In the mouse, McDonnell et al., (2008) showed that exogenous ATP relaxed the IAS. Under non-adrenergic, non-cholinergic conditions, purinergic pathways contribute to EFS-induced inhibitory junction potentials and relaxation in addition to the nitrergic pathways of the mouse IAS (Duffy et al., 2012). P2 receptor antagonists significantly reduced the fast inhibitory junction potential and relaxation, achieved by desensitisation of P2Y receptors with αβmATP and by the selective P2Y1 receptor blocker and K^+ channels blocker (McDonnell et al., 2008). Thus, purinergic transmission significantly contributes to NOS-independent neural inhibition in the mouse IAS.

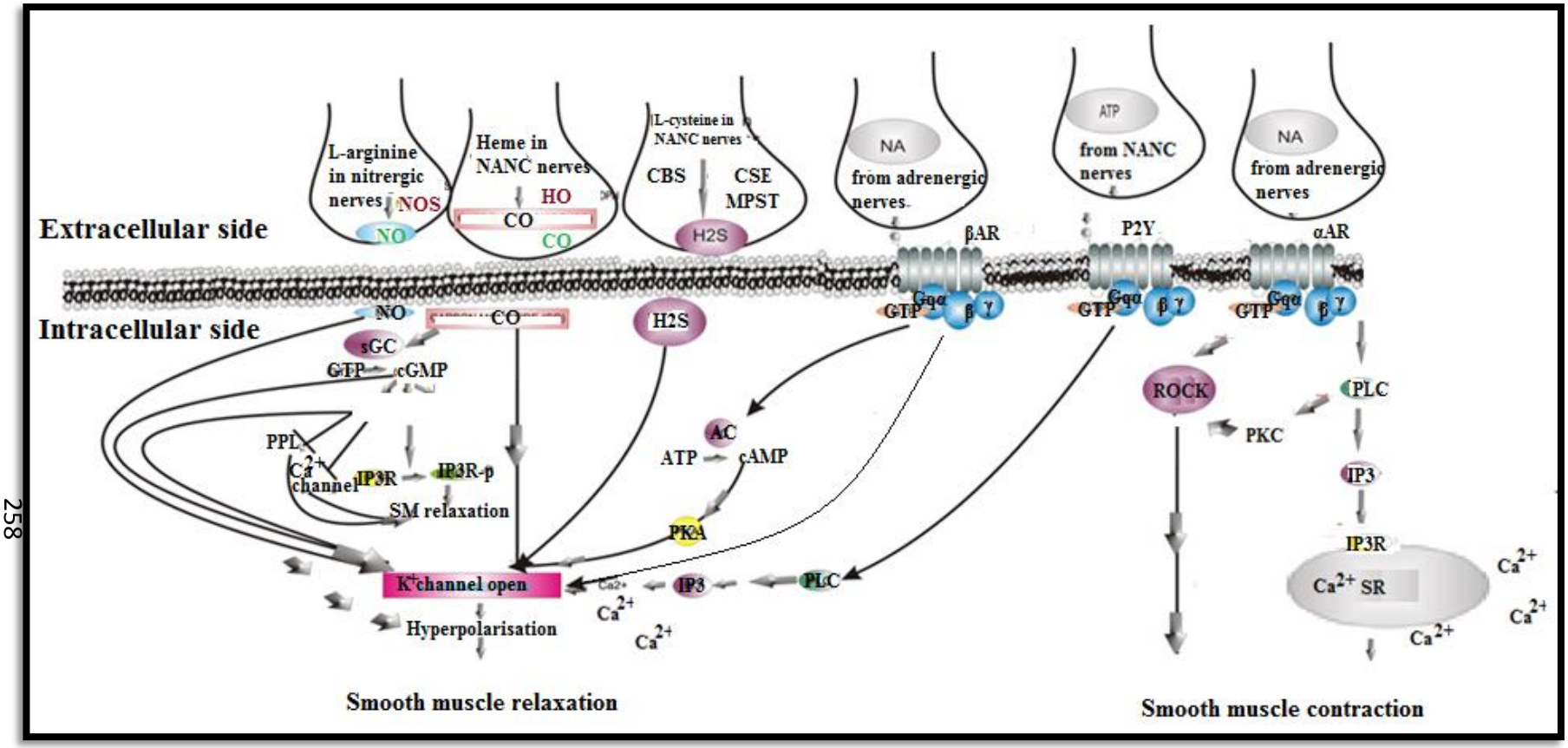


Figure 8.1 Potential pathways for smooth muscle regulation/control in the anorectum. Nitric oxide released by the nitrergic nerves can directly induce smooth muscle relaxation by activation of guanylate cyclase (GC) which converts GTP to cyclic GMP leading to smooth muscle relaxation. On the other hand, carbon monoxide (CO), can induce smooth muscle relaxation by opening K^+ channels or by activating the guanylate cyclase. In addition, H_2S can induce smooth muscle relaxation by opening K^+ channels. Finally, ATP, cyclic AMP or adenosine may also induce smooth muscle relaxation. Contraction occurs via increase in intracellular Ca^{2+} release and Rho kinase (ROCK). (NANC: non-adrenergic, non-cholinergic, GC: guanylate cyclase, HO: heme oxygenase, NOS: nitric oxide synthase, NO: nitric oxide, PDE: phosphodiesterase, PKG: cGMP dependent protein kinase, PPL: phospholamban, IP_3R: IP_3 receptor, CBS cystathionine –β-synthetase, CSE: cystathionine-γ-lyase, MPST: 3-mercaptosulfutransferase, AC: adenylate cyclase) (Extracted from Denninger & Marletta, 1999, Francis et al., 2010, Koh et al., 1997, Rattan et al., 2015).

Also, ATP has been shown to contribute to inhibitory junction potentials and relaxation in conjunction with NO in other animals such as rat (De Luca et al., 1999; Opazo et al., 2011), guinea-pig (Rae & Muir, 1996) and human (Burleigh et al., 1979). In the IAS of rats, ATP is co-released by inhibitory motor neurones with NO and VIP, where it could induce IAS relaxation (Opazo et al., 2011). In the gastrointestinal tract, activated purinergic receptors induce an increase in intracellular Ca^{2+}, and the opening of small conductance Ca^{2+}-activated K^+ channels, with an ultimate effect of hyperpolarization and relaxation (Koh et al., 1997; Kong et al., 2000; Vogalis & Goyal, 1997).

Carbon monoxide (CO)

Carbon monoxide synthesis is catalysed by the heme oxygenase, which is the rate-limiting enzyme in the catabolism of heme. Heme oxygenase degrades heme, producing CO, biliverdin, and Fe^{2+} (Tenhunen et al., 1969). There are two isoforms of heme oxygenase (heme oxygenase-1 and heme oxygenase-2) of which heme oxygenase-1 is an inducible enzyme, whereas heme oxygenase-2 is constitutively expressed.

Non-adrenergic, non-cholinergic neurotransmission is markedly decreased in the upper gastrointestinal tract of heme oxygenase knockout mouse, but can be restored by addition of exogenous CO (Xue et al., 2000). Moreover, CO and NO are thought to be co-transmitters in the gastrointestinal tract, since CO restores intestinal smooth muscle relaxation in heme oxygenase knockout mice (Xue et al., 2000).

Carbon monoxide also induces activation of guanylate cyclase (Verma et al., 1993). The guanylate cyclase activated by CO induces production of cyclic GMP from GTP, the opening of K^+ channels such as voltage-gated K^+ channels (Kv), with hyperpolarisation resulting in smooth muscle relaxation (Figure 8.1; Farrugia et al., 1993). Carbon monoxide-releasing molecules, CO, as well as non-adrenergic, non-cholinergic nerve stimulation, produce IAS relaxation via guanylate cyclase/cGKI activation (Rattan et al., 2004). Moreover, CO-induced smooth muscle relaxation has been reported via a guanylate cyclase-independent pathway, a process by which CO opens K^+ channels to cause hyperpolarization and smooth muscle relaxation (Figure 8.1) (Rich et al., 1994; Wang & Wu, 1997; Wang et al., 1997).

Vasoactive intestinal polypeptide (VIP)

Vasoactive intestinal polypeptide, originally isolated from pig intestinal extracts by Said & Mutt (1970), has also been shown to participate in non-adrenergic, non-cholinergic relaxation throughout the intestine, including the IAS of animal (Biancani et al., 1985; Keef et al., 2013) and human (Burleigh et al., 1979; Raghavan et al., 2011). Keef et al., (2013) showed that during longer EFS trains, a non-purinergic, non-nitrergic relaxation and hyperpolarization developed slowly and persisted for several minutes beyond the end of the stimulus train, which was abolished by a VIP receptor antagonist, was absent in VIP knockout mice and was mimicked by exogenous VIP. Thus, suggesting the contribution of VIP in inhibitory neuromuscular transmission in the mouse IAS (Keef et al., 2013). Keef et al., (2013) suggested that in vivo the VIP pathway may be activated with greater rectal distensions, leading to a more prolonged period of anal relaxation.

Using genetic knockout of the biosynthetic enzymes for CO and NO in mice, Watkins et al., (2004) showed that the physiologic effects of exogenous and endogenous VIP in the mouse IAS are mediated by heme oxygenase-2-synthesized CO (Watkins et al., 2004). Immunoantagonism of VIP blocked non-adrenergic, non-cholinergic transmission in the lower esophageal sphincter of the opossum (Goyal et al., 1980), exogenous VIP mimicked non-adrenergic, non-cholinergic transmission, and VIP is localized to myenteric plexus neurons that mediate non-adrenergic, non-cholinergic transmission in the lower esophagus of cat and pig (Uddman et al., 1978). At the postsynaptic level, many studies support that VIP and NO are parallel co-transmitters, acting via the adenylate cyclase and guanylate cyclase pathways respectively. Based on results from gastrointestinal tract smooth muscle cells, VIP is thought to be the principal neurotransmitter, acting partly via a VIP receptor and the adenylate cyclase/cyclic AMP pathway and by induction of muscular NO production (Van Geldre & Lefebvre, 2004). In addition, the capacity of VIP to release NO from isolated smooth muscle cells is associated with the induction of iNOS in the cells (Van Geldre & Lefebvre, 2004). Thus, VIP may mediate relaxation of the IAS by a direct effect or via modulation of other non-adrenergic, non-cholinergic neurotransmitters such as NO and CO.

Hydrogen Sulphide (H_2S)

Hydrogen sulphide is the third known gasotransmitter and the most recent candidate to join the family of gasotransmitters. It is a small molecule; that is more soluble in a lipophilic solvent with high membrane permeability (Chen et al., 2007). Hydrogen sulphide is produced by non-adrenergic, non-cholinergic, nerves from L-cysteine by two enzymes; cystathionine-β-synthethase and cystathionine-γ-lyase, although, a third enzyme (3-mercaptosulfutransferase) has been associated with H_2S synthesis in mammals (Hosoki et al., 1997). Expression of these enzymes is widespread throughout the body, but particularly in the liver and brain (Kabil et al., 2011). Hydrogen sulphide has been implicated in K^+ channels opening and hyperpolarization. Among the K^+ channels opened by H_2S, SK, ATP-dependent K^+ (KATP), voltage-dependent K^+ (Kv) and inwardly rectifying K^+ channels (Kir) have been suggested (Kubo et al., 2007; Zhao et al., 2001; Pouokam & Diener, 2011; Takir et al., 2015). However, H_2S relaxations could also be achieved without K^+ channels opening and may involve modulation of Ca^{2+} sensitization as shown in the mouse distal colon (Dhaese et al., 2010).

Teague et al., (2002) reported that NaHS (H_2S donor) relaxed isolated rabbit ileum and acetylcholine-mediated contraction of the isolated guinea-pig ileum. In mouse gastric fundus H_2S causes relaxation partially via activation of myosin light chain phosphatase (Dhaese & Lefebvre, 2009). Using cystathionine-β-synthase knockout mice and immuno-technique, H_2S has been shown to modulate gastric compliance (Xiao et al., 2015). In addition, both cystathionine-β-synthethase and cystathionine-γ-lyase have been localised along the length of rat and mouse colon (Gil et al., 2011; Martin et al., 2010). Linden et al., (2008) demonstrated the expression of messenger RNA and protein localisation for cystathionine-β-synthethase and cystathionine-γ-lyase in the mouse colon. No studies have yet investigated whether H_2S is an inhibitory transmitter in the IAS, however, H_2S may mediate the relaxation of IAS.

Acetylcholine

Acetylcholine which acts via the muscarinic receptors has been shown to be excitatory and inhibitory in the IAS smooth muscle. For example, excitatory neurotransmission due to acetylcholine was shown in the mouse (Duffy et al., 2012) and human (Somara et al., 2009)

IAS. However, in the cat, acetylcholine induced relaxation of the IAS to a major extent via an activation of nitrergic, inhibitory motor neurones (Buntzen et al., 1996). In human, mAChR activation seems to mediate the release of inhibitory neurotransmitters, which are not prostaglandin, histamine, 5-hydroxytryptamine, or dopamine, but could be ATP or VIP (Burleigh et al., 1979). Thus, activation of mAChR may mediate the inhibitory junctional potentials and relaxation by mediating the release of relaxing neurotransmitters. The contractile effect to acetylcholine reported in mouse and human IAS may be due to the low amount of relaxing agent release in response to mAChR activation (Duffy et al., 2012; Somara et al., 2009). Thus, relaxation was masked by the contractile action. The neurotransmitters involve in the modulation of the IAS smooth muscle is summarised in Table 8.1.

Thus, the literature concerning the neurotransmitters of the IAS is confusing. A number of contracting and relaxing transmitters have been proposed but even the response to the classical transmitters such as acetylcholine is unclear. The aim of this chapter is, therefore, to characterise the excitatory and inhibitory neurotransmitters involved in control of the porcine IAS. In particular, this study will examine whether a number of inhibitory neurotransmitters can be shown to be co-released in one species.

8.3 MATERIALS AND METHODS

Strips of female pig IAS smooth muscle were set up in organ baths as described in chapter 2. During the 60 minutes equilibration, a basal resting tone consistently developed in these tissues (Figure 8.2). Thus tension was adjusted over the 60 minutes period until stable at 2g.

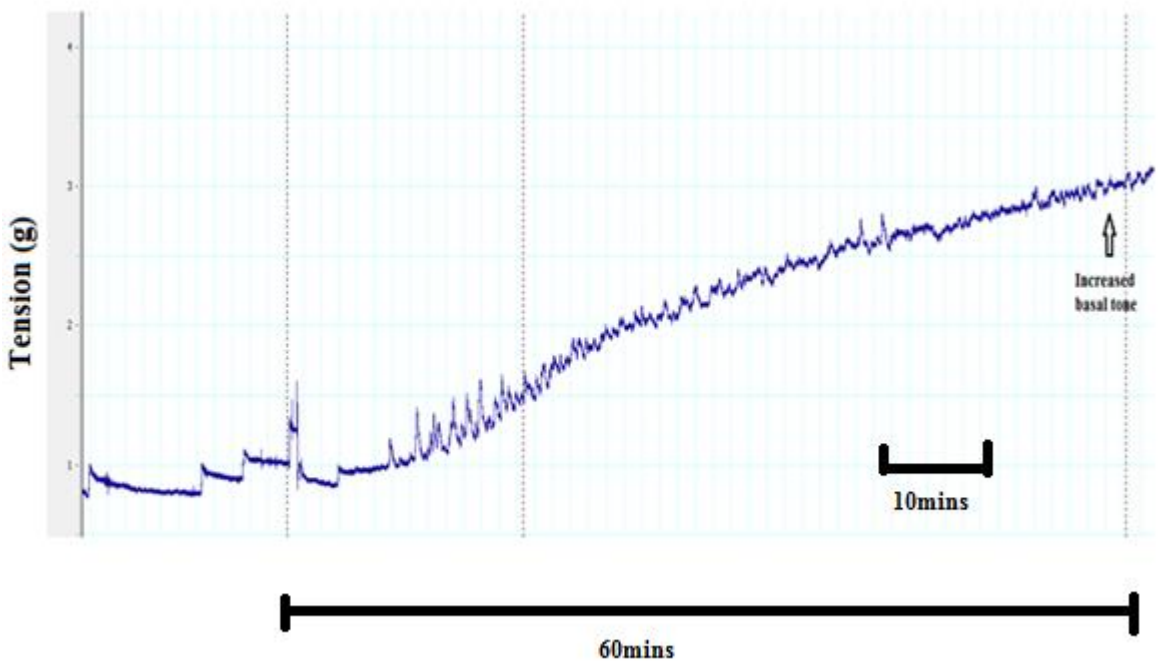


Figure 8.2 Recording illustrating the increase in the basal tone of the porcine internal anal sphincter strips during equilibration (1g= 100mN).

Electrical field stimulation (EFS)

The smooth muscle strips were electrically stimulated as described in chapter 2. Initial responses for each frequency were obtained followed by responses in the presence of one of the drugs which interfere with neurotransmission. Antagonists/inhibitors were equilibrated with tissues for 30 minutes before obtaining responses to EFS. Only one drug was tested in each tissue and control experiments confirmed responses to EFS did not change over this short time period. Guanethidine (10μM) was used to block transmitter release from the sympathetic nerves, atropine (1μM) to block mAChR and α, β-methylene-ATP (10μM) to desensitise P2X receptors. Also, L-NNA (100μM), indomethacin (5μM), PAG

(propargylglycine; 1mM), AOAA (aminooxyacetic acid; 30μM), PheLeu-VIP (100nM), ODQ (10μM), ZnPPIX (10μM), suramin (100μM), and 8-phenyltheophyline (10μM) were used to block NOS, cyclooxygenase, cystathionine-γ-lyase, cystathionine-β-synthethase, VIP receptor, guanylate cyclase, heme oxygenase, P2 receptor and P1 receptor respectively. Moreover, TTX (1μM), a potent neurotoxin and selective inhibitor of neuronal sodium channel conductance was used to confirm that the smooth muscles were not directly activated using these stimulation parameters.

Statistical Methods

Changes in developed tension were expressed and the responses seen in the presence of antagonist/inhibitor were compared as described in chapter 2.

8.4 RESULTS

Stimulation of the IAS strips via EFS induced a biphasic response, a relaxation response followed by a contraction response, as shown in Figure 8.3A&B. At 5 Hz, the relaxation was fast, lasting 2-3 seconds, followed by a contraction of approximately 10 seconds duration (Figure 8.3A). Stimulation at a frequency of 10 Hz produced a contraction that was greatly increased in both size and duration (Figure 8.3C). The responses were neurogenic in origin, and the neurotoxin tetrodotoxin (1μM, n = 8) completely abolished contractions at both frequencies. Relaxations to EFS were also greatly reduced by tetrodotoxin (3 μM) by 78.3 ± 2.8% and 91.5 ±1.6% at 5Hz and 10Hz respectively (both $P < 0.001$).

Contractile Responses to EFS

Removal of the adrenergic component with guanethidine (10μM) almost completely abolished the contraction of the IAS to electrical stimulation at both frequencies (Figure 8.3B). Guanethidine reduced the large contractile responses at 5Hz and 10 Hz to contractions that were superimposed on large relaxations and only recovered to 30% and 20% below the baseline respectively ($n=7$, $p<0.01$; Figure 8.3B; Table 8.2). The percentage decrease in contractile response after incubation with guanethidine was 130.7±47.2% and 120.0±18.7% for 5Hz and 10Hz respectively. Contractile responses were also reduced following desensitisation of P2X receptors with the potent purinoceptor agonist α, β-mATP (10 μM) (Figure 8.3D; Table 8.2). Responses at both frequencies were reduced, but the effect was only statistically significant for responses at 5 Hz ($n=4$, $p<0.01$; Table 8.2). Desensitisation of ATP receptors with α, β-mATP, decreased the contractile response by 44.8±5.0% and 20.4±15.1% at 5Hz and 10Hz respectively. In contrast, responses to electrical stimulation were not significantly altered by the presence of the mAChR antagonist atropine (1 μM, Table 8.2).

Relaxation Responses to EFS

The relaxation responses obtained after removing adrenergic, cholinergic and purinergic contractions with guanethidine, atropine and α, β-mATP were also examined in greater detail to determine which inhibitory neurotransmitters were involved and their relative importance.

Under these conditions, relaxations to EFS were unaffected by the cyclooxygenase inhibitor indomethacin (5μM, n = 14) or the VIP- receptor antagonist PheLeu-VIP (100nM, n =6; Table 8.3). However, in the presence of the NO synthase inhibitor L-NNA (100μM), relaxations were reduced by 40-50% (Figure 8.4; Table 8.3). Inhibition of guanylate cyclase with ODQ (10μM) produced a greater inhibition than L-NNA (IAS relaxation response by 70.9 ±6.7% and 62.5±5.3% at 5Hz and 10Hz respectively; n=7-8, $p<0.001$), but a combined L-NNA/ODQ treatment did not produce a greater inhibition of relaxation than ODQ alone (Figure 8.4).

In another series of experiments the non-nitrergic relaxation remaining in the presence of L-NNA (100μM) was investigated further. As previously, these relaxation experiments were performed in the presence of guanethidine, atropine and α, β-mATP to remove contractile responses. The relaxations obtained in the additional presence of L-NNA (100μM) were not affected by either the adenosine receptor (P1) antagonist 8-phenyltheophyline (10μM, n = 5) nor the P2-purinoceptor antagonist suramin (100μM, n = 5). The possible contribution to relaxation by CO was examined using zinc protoporphyrin IX (ZnPPIX, 10μM), which inhibits the synthesis of CO by heme oxygenase, and the contribution to relaxation by H_2S was examined using a combination of propargylglycine (PAG,1mM) and aminooxyacetic acid (AOAA, 30μM) to inhibit H_2S synthesis. The inhibition of CO synthesis reduced relaxation of the IAS to EFS by 42.0±7.2 and 21.6±3.6% at 5Hz and 10Hz respectively (n=10-11, $p<0.01$, $p<0.001$) and H_2S synthesis (relaxation of the IAS to EFS by 26.7±5.8% and 20.9±3.1% at 5Hz and 10Hz respectively) (Figure 8.5). The inhibition of guanylate cyclase with ODQ reduced relaxations by about 70% and removing H_2S with PAG/AOAA further reduced relaxation responses producing greater inhibition than ODQ alone. In contrast, the effects of CO removal with ZnPPIX were not additive with ODQ, the combination of ZnPPIX/ODQ yielding the same reduction in relaxation to EFS as ODQ alone (Figure 8.5).

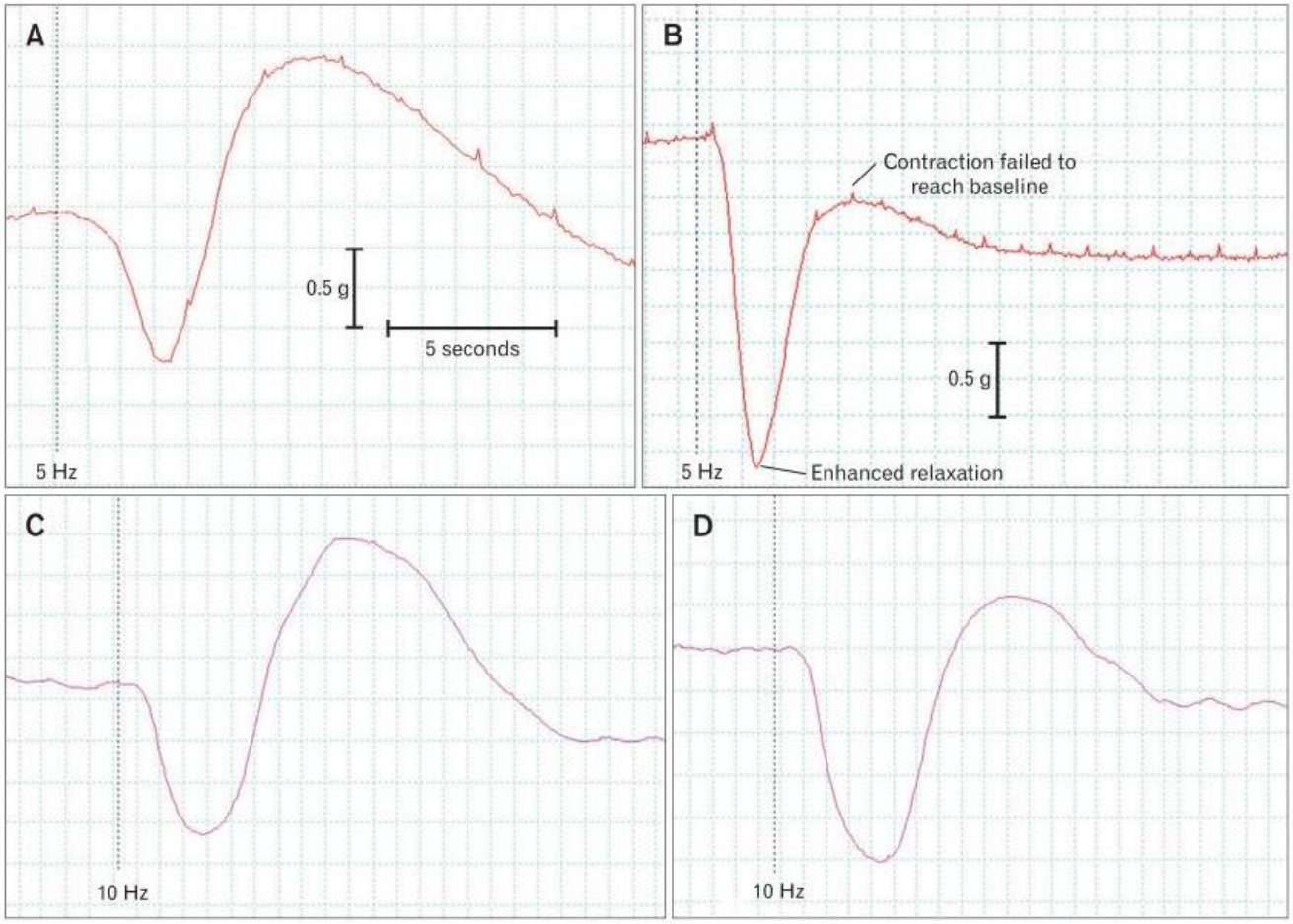


Figure 8.3 Representative responses of internal anal sphincter strips to electrical field stimulation in the absence (A & C) or presence of the adrenergic neurone blocker guanethidine (10μM, B) or after desensitisation of P2 receptors with α, β-mATP (10μM, D) (1g= 100mN).

Drug	Frequency	Response (% of baseline)		Inhibition (% of control response)
		Absence of drug	Presence of drug	
Guanethidine (10µM) n=7	**5Hz**	21.5±10.3	**-9.0±4.8 ****	130.7±47.2
	10HZ	55.7±17.3	**-8.2±9.1 ****	120.0±18.7
Atropine (1µM), n=8	**5Hz**	31.1±6.1	24.2±6.2	10.0±20.8
	10HZ	46.4±9.2	35.3±7.3	13.6 ± 20.2
α,β-mATP (10µM), n=4	**5Hz**	33.4±9.6	**19.2±7.4 ****	44.8±5.0
	10HZ	53.8±17.7	4.5±12.9	20.4 ± 15.1

Table 8.2 Mean (± SEM) contractions developed by tissues in response to electrical field stimulation in the presence or absence of inhibitors. n=4-7, **p<0.01 vs. control responses in the absence of drug (Paired Student's t-test).

		Relaxation (% of baseline)		Inhibition (% of control response)
Drug	**Frequency**	**Absence of drug**	**Presence of drug**	
L-NNA (100µM), n=26	**5Hz**	56.6%±2.2	**32.8%±2.8 *****	**49.9 ± 5.2**
	10Hz	45.4%±3.7	**19.9%±2.9 *****	**40.3 ± 5.9**
Atropine (1µM), n=8	**5Hz**	54.8%±10.0	63.1%±6.2	No change
	10Hz	58.6%±10.1	64.0%±6.5	No change
α,β-mATP (10µM), n=4	**5Hz**	26.1%±13.1	**33.3%±12.3 ***	**Increased**
	10Hz	20.9%±12.7	**30.9%±14.1 ***	**Increased**
Indomethacin (5µM), n=14	**5Hz**	44.9%±3.7	44.0%±3.6	No change
	10Hz	47.1%±4.2	45.4%±4.5	No change
PheLeu-VIP (100nM), n=6	**5Hz**	50.0±4.2	48.0±3.9	No change
	10Hz	52.6±4.9	52.0±4.3	No change
8-Phenyltheophyline (10µM), n=5	**5Hz**	42.9±6.9	47.5±7.1	No change
	10Hz	47.0±6.8	39.0±9.0	No change
Suramin (100µM), n=5	**5Hz**	38.6±4.6	45.2±4.6	No change
	10Hz	39.5±7.7	48.6±5.0	No change

Table 8.3 Mean (± SEM) relaxation responses expressed as a percentage of the tone of the tissue at the time of stimulation in the presence or absence of inhibitors. n=4-26, * P<0.05, and ***P<0.001 vs. control responses in the absence of drug (Paired Student's t-test).

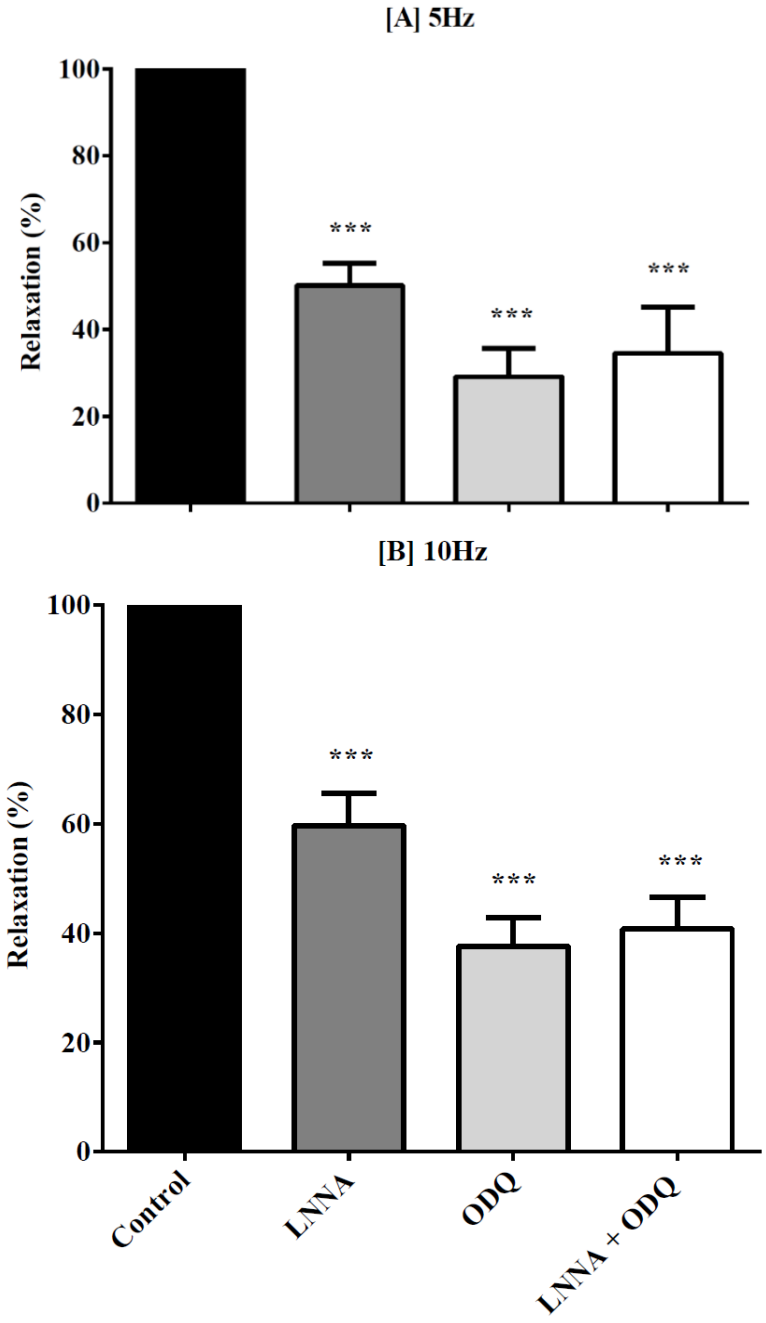


Figure 8.4 Relaxation responses to electrical field stimulation in the presence or absence of inhibitors of nitric oxide production (L-NNA) and guanylate cyclase (ODQ). n=5-8. *** P<0.001 compared to control values in the absence of inhibitors (one way ANOVA with Tukey post-hoc test).

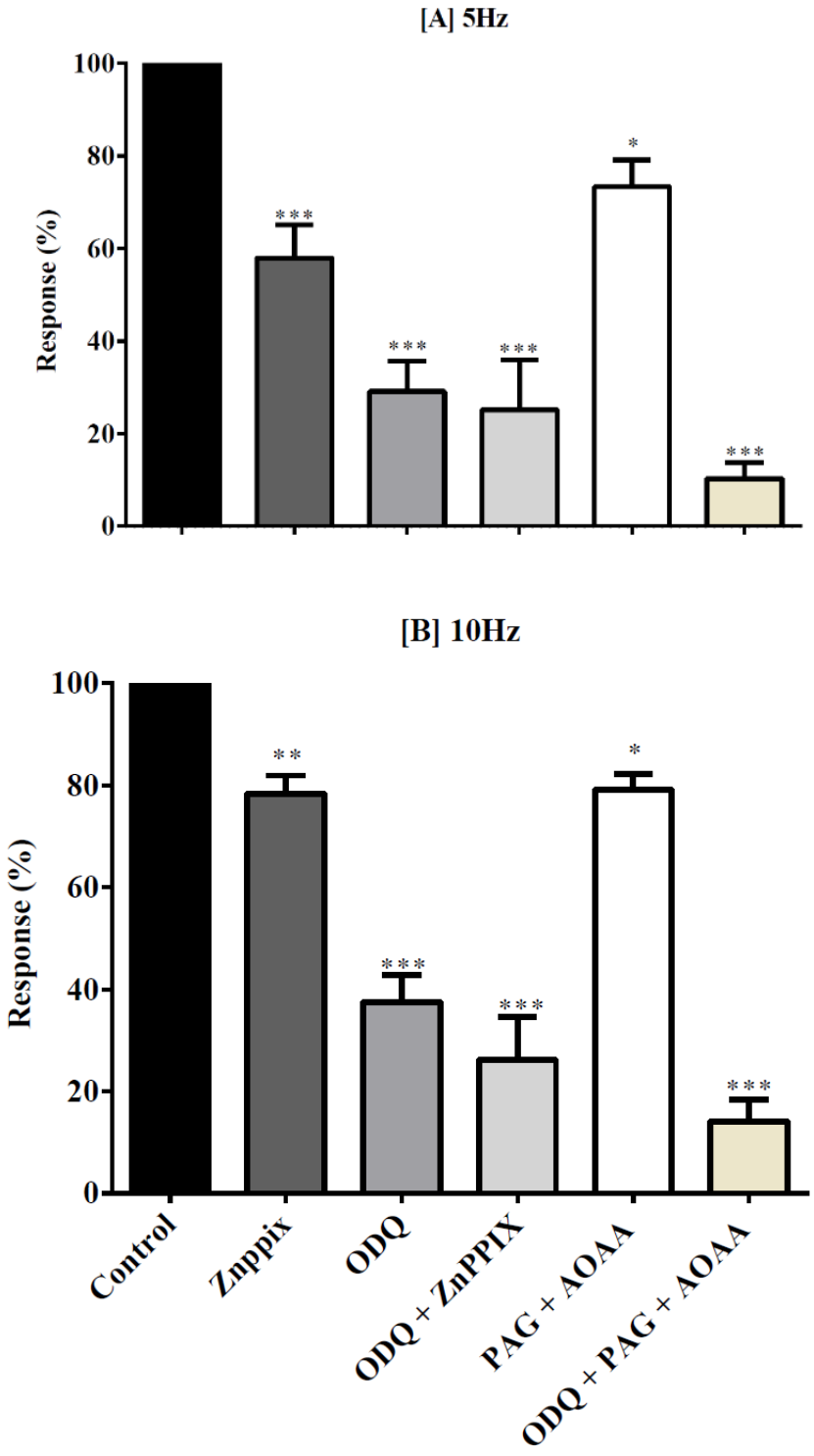


Figure 8.5 Relaxation responses to electrical field stimulation in the presence or absence of inhibitors of carbon monoxide (ZnPPIX), hydrogen sulphide production (PAG + AOAA) and guanylate cyclase (ODQ). n=5-11. *P<0.05, ** P<0.01, and *** P<0.001 compared to control values in the absence of inhibitors (one way ANOVA with Tukey post-hoc test).

8.5 DISCUSSION

Which transmitters contribute to internal anal sphincter contraction to electrical field stimulation?

Stimulation of the porcine IAS strips via EFS induced a relaxation response followed by a contraction response, and first, we investigated the contractile neurotransmitter responsible for the contractile component of the response.

Electrical field stimulation produced initial contractions of the IAS, which were blocked by the α-AR antagonist in mouse (Cobine et al., 2007), sheep (Acheson et al., 2009) and humans (Speakman et al., 1990; 1993). Thus, it is known that there is an α-adrenergic excitatory component of the response to EFS in the IAS smooth muscle. Acetylcholine may also mediate the contractile function of the IAS as shown in the mouse (Duffy et al., 2012) and human (Somara et al., 2009). In the bladder, ATP activates P2X, as confirmed by organ bath experiments and immunostaining (Lee et al., 2000) and $P2X_1$ knockout mice (Vial & Evans, 2000). There appear to be species-dependent differences in the innervation of the IAS as shown in the monkey, rabbit or mouse (Cobine et al., 2007). In the monkey, the IAS is functionally innervated by sympathetic nerves that contract the IAS via excitatory α-ARs. In contrast, no significant motor function was identified for sympathetic nerves in the mouse or rabbit IAS (Cobine et al., 2007). To our knowledge, no studies have shown the contractile neurotransmitters released by the IAS smooth muscle of the pig in response to EFS even though it is the most relevant model for human.

In this study, guanethidine was used to investigate the role of noradrenaline in the contraction of the pig IAS smooth muscle. Guanethidine is a neurotoxin selective for adrenergic nerves and abolishes adrenergic nerve transmission completely at a concentration of 10μM (Cobine et al., 2007). Guanethidine depressed contractions completely from a large contraction above the baseline to one that failed to increase to the baseline level following the initial relaxation response, suggesting a prolonged relaxation mechanism. Thus, guanethidine inhibited contraction by >100%, which prevented an accurate calculation of the contribution of noradrenaline to contraction of the IAS.

Although the exact contribution of noradrenaline could not be calculated, it can be concluded that noradrenaline is the main excitatory neurotransmitter released in the IAS. The result of this study supports previous reports in the literature. Acheson et al., (2009) showed in the sheep IAS smooth muscle that noradrenaline acts via post-junctional α-AR to antagonise neurogenic relaxations. Also in the monkey, the IAS smooth muscle is functionally innervated by sympathetic nerves that contract the muscle via excitatory α-ARs (Cobine et al., 2007). In human, there is an α-adrenergic excitatory component of the response to EFS of the intramural nerves which was blocked by an α-AR inhibitor (Speakman et al., 1990; 1993). In previous studies, our group has shown that these adrenergic responses are mediated via the $\alpha_{1A/L}$-AR in the porcine IAS (Mills et al., 2008).

In the present study desensitisation of P2X receptors also depressed contractile responses to EFS indicating an excitatory role for ATP in this tissue. ATP is often released as a co-transmitter from autonomic nerves and is released with noradrenaline in many tissues including the colon (Venkova & Krier, 1993) and vasculature (Racchi et al., 1999). These previous studies used the agonist α, β-mATP to desensitise P2X purinergic receptors and it was used in the present study to desensitise the purinergic receptors of the IAS. It depressed contractile responses to EFS, although the inhibition was significantly less than that produced by guanethidine. The decrease in contraction to EFS by α, β-mATP was greater at 5Hz stimulation than at 10Hz suggesting that ATP provides a greater contribution to contractions at lower frequencies of stimulation. This idea is supported by the report that the inhibition of ATP breakdown has a greater potentiating effect on the neurogenic responses of visceral and vascular smooth muscle at low frequencies compared to higher frequencies (Khurana et al., 2004). Thus the present results suggest a role for ATP in the neurogenic contractions of the IAS smooth muscle via P2X receptors, at least at lower frequencies of stimulation. Surprisingly, previous studies have suggested that ATP is an inhibitory neurotransmitter in the IAS (Opazo et al., 2011; Rae & Muir, 1996; Rattan, 2005). ATP mediates inhibitory junction potentials and relaxations in the guinea-pig (Rae & Muir, 1996) and human (Burleigh et al., 1979) IAS. Both ATP and adenosine have been shown to produce concentration-dependent relaxations of the guinea pig (Crema et al., 1983; Rae & Muir, 1996), rabbit (Biancani et al., 1985), rat (Nissan et al., 1984; Opazo et al., 2011), mouse (Keef et al., 2013) and sheep (Acheson et al., 2009) IAS smooth muscle. The inhibitory

junction potentials mediated by ATP was through $P2Y_1$ receptors in the rat (Opazo et al., 2011), mouse (Keef et al., 2013) and sheep (Acheson et al., 2009) IAS. Thus, P2X receptors may mediate contractile responses of the IAS whilst the P2Y receptors may mediate the inhibitory junction potentials and relaxations (Acheson et al., 2009; Keef et al., 2013; Opazo et al., 2011). In other tissues such as the bladder, ATP can induce smooth muscle relaxation via three possible mechanisms (1) directly via P2Y receptors (2) indirectly by inducing NO release from epithelial cells and (3) indirectly via P1 receptors after being broken down to adenosine (Hernandez et al., 2009; McMurray et al., 1998; Vesela et al., 2012). However, in the current studies the mucosa had been removed before tissues were set up and neither the P2-receptor antagonist suramin nor the P1-receptor antagonist 8-phenyltheophyline had any effect on relaxations, thus ruling out a role for ATP in relaxation responses of this tissue.

Which transmitters contribute to relaxation to EFS?

Using EFS and pharmacological agents, several studies have shown that the inhibitory neurotransmission in the IAS is mainly via NO in animals (Cobine et al., 2014; Mundey et al., 2000; Opazo et al., 2011; Ratan & Chakder, 1992) and human (O'Kelly et al., 1993b). Neurogenic relaxations of the IAS induced by EFS were significantly reduced in the presence of a NOS inhibitor suggesting the major involvement of NO as a neurotransmitter in the sheep (Acheson et al., 2009), and mouse (McDonnell et al., 2008). However, other neurotransmitters have been associated with the inhibitory junctional potential and relaxation of the IAS. For example, EFS produced a residual non-nitrergic, non-adrenergic, apamin-sensitive relaxation which was inhibited by a P2Y(1) receptor antagonist suggesting that purinergic transmission significantly contributes to NOS-independent neural inhibition in the sheep (Acheson et al., 2009) and mouse IAS (McDonnell et al., 2008). Thus, we hypothesised that the NO might be the major inhibitory neurotransmitter in the porcine IAS. Moreover, other neurotransmitters may contribute to the inhibitory junctional potential and relaxation of the porcine IAS. To our knowledge, no study has yet analysed the relaxing neurotransmitter in the porcine IAS in response to EFS.

In this study, after inhibition of the contractile responses, relaxation responses to EFS were greatly enhanced and the mechanisms involved were investigated in more detail. The NOS inhibitor L-NNA (100μM) reduced the relaxation induced by field stimulation by

approximately 40-60%, suggesting that NO is responsible for approximately half of the relaxation response of the sphincter to nerve stimulation. This result supports literature showing that inhibitory junctional potentials and relaxation of the IAS is mainly mediated by NO (Mundey et al., 2000; Opazo et al., 2011; Rattan & Chakder, 1992). In the opossum, NO caused a concentration-dependent fall in the resting tone of the IAS, and the inhibitory action of NO may also be exerted directly on the IAS smooth muscle since it was not modified by the neurotoxin tetrodotoxin, which abolished the neurally mediated decrease in the IAS tension (Ratan & Chakder, 1992). In the human IAS, an exogenous donor of NO relaxed tissue strips in a concentration-dependent manner and in the presence of atropine and guanethidine, transmural field stimulation produced tetrodotoxin-sensitive relaxations, which were inhibited in a dose-dependent manner by inhibitors of NOS (O'Kelly et al., 1993b). In the mouse, nitrergic relaxation depended on cyclic GMP-dependent protein kinase I and inhibitors of NOS abolished responses which confirm an essential role for guanylate cyclase in NO-induced relaxation of the IAS (Cobine et al., 2014). Thus, the NO-dependent inhibitory junctional potentials and relaxation may be mediated via guanylate cyclase in the pig.

The intramuscular ICC contribute to the electrical events underlying nitrergic neuromuscular transmission in the mouse IAS (Duffy et al., 2012) and ICCs have been observed in the IAS of mouse (Cobine et al., 2011; 2014; Duffy et al., 2012), monkey (Cobine et al., 2010) and humans (Lorenzi et al., 2014; Piotrowska et al., 2003). Thus, ICC may contribute to the NO-induced inhibitory junctional potential and relaxation in the pig IAS. However, to our knowledge, ICC have not been reported in the porcine IAS.

Other non-nitrergic inhibitory neurotransmitters proposed in the IAS include acetylcholine, VIP, CO, and H_2S. For example, VIP has been shown to participate in the inhibitory neuromuscular transmission of the IAS of rabbit, mice (Biancani et al., 1985; Keef et al., 2013) and human (Burleigh et al., 1979; Raghavan et al., 2011). Also, CO directly relaxes the mouse IAS (Rattan et al., 2005). Using a heme oxygenase knockout mouse, Xue et al., (2000) showed that responses to EFS are nearly abolished in these animals despite persistent nNOS expression, whereas exogenous CO restores normal responses, indicating that the NO system might not function in the absence of CO generation. Carbon monoxide produced IAS relaxation via guanylate cyclase/cGKI activation in the rat IAS smooth muscle (Rattan et al.,

2004). Also, H_2S production has been demonstrated in the gastrointestinal tract, and both cystathionine-β-synthethase and cystathionine-γ-lyase have been localised along the rat and mouse intestine (Gil et al., 2011; Martin et al., 2010). To our knowledge expression of H_2S has not be shown in the IAS. Hydrogen sulphide is endogenously produced in the rat colon where it regulates colonic motility (Gil et al., 2011). Cystathionine-β-synthethase and cystathionine-γ-lyase enzymes have been detected immunohistochemically in rat colonic smooth muscle cells (Hennig & Diener, 2009). Linden et al., (2008) demonstrated the expression of messenger RNA and protein localisation for cystathionine-β-synthethase and cystathionine-γ-lyase in the colon, brain and liver. Moreover, they showed that expression levels of the enzymes vary between tissues and are differentially distributed.

Previous studies have suggested that mAChR stimulation results in relaxation which appears to be mediated via the release of NO in the cat, monkey (Buntzen et al., 1996; Rayner, 1979) and human (Burleigh et al., 1979; O'Kelly et al., 1993a). In the present study mAChR stimulation with carbachol elicited contractions and no relaxation was observed. Furthermore, atropine had no effect on the excitatory responses to EFS, and the inhibitory responses were obtained in the presence of atropine, ruling out a contribution from acetylcholine. In other tissues, such as blood vessels mAChR stimulation of epithelial cells can release NO to induce relaxation (Khurana et al., 2004). Similarly, in the urinary bladder, mAChRs are responsible for direct smooth muscle contraction (Hegde, 2006), but they may also stimulate the release of NO from urothelial cells (Andersson et al., 2008; Kullmann et al., 2008a). In the present study experiments were performed on muscle strips with the mucosa removed to allow neurotransmission to be examined without the complication of epithelial influences and no relaxation responses were observed.

To determine the other transmitters contributing to IAS relaxation, the non-nitrergic relaxation induced by EFS in the presence of L-NNA was also investigated. Neither the VIP receptor antagonist PheLeu-VIP nor the cyclooxygenase inhibitor indomethacin significantly reduce the non-nitrergic relaxation ruling out a role for VIP or prostaglandins in relaxation of the pig IAS, a conclusion also reached by Acheson et al., (2009) in the sheep IAS. Also, Burleigh et al., (1979) discounted prostaglandin E_2 and prostaglandin $F_{2\alpha}$ as possible neurotransmitters of the non-cholinergic, non-adrenergic inhibitory nerves in the human IAS. However, the guanylate cyclase inhibitor ODQ (Dellabianca et al., 2007) did have a

significant effect, reducing relaxations by approximately 70%. Carbon monoxide, which mediates smooth muscle relaxation responses via cyclic GMP, is an inhibitory neurotransmitter that plays a role in the relaxation of the opossum sphincter (Rattan et al., 2004). Carbon monoxide caused a concentration-dependent and tetrodotoxin-resistant fall in the resting tension of the opossum IAS and caused an increase in the tissue cyclic GMP levels (Rattan & Chakder, 1993). Also, the opossum IAS smooth muscle was found to have significant levels of basal heme oxygenase activity (Rattan & Chakder, 2000). Using immunocytochemistry in the same animal, the enzyme heme oxygenase, which produces CO, has been found to be present in the myenteric and submucosal neurones of the IAS as well as in the ICCs found in this tissue (Battish et al., 2000). The non-nitrergic relaxation of the pig IAS to field stimulation was greatly reduced by ODQ which suggests the involvement of CO in the relaxation of this tissue. This was confirmed using ZnPPIX an inhibitor of heme oxygenase which is responsible for the synthesis of CO. This inhibitor reduced IAS relaxation responses by about 20% and this effect was not additive with ODQ. These data suggest that the ODQ sensitive component of the relaxation is composed of predominantly NO (50%) but with a smaller contribution from CO (20%).

In the presence of ODQ, the remaining small, non-NO, non-CO relaxation must be mediated via a neurotransmitter that is cyclic GMP-independent. It has been shown that the enzymes responsible for the production of H_2S are present in the enteric nerves and the myenteric ICC (Schicho et al., 2006), both of which can be found in the IAS (Shafik et al., 2006). Inhibition of H_2S synthesis with PAG/AOAA depressed relaxation responses to EFS and the depression was additive to that of ODQ and therefore independent of cyclic GMP indicating a small role for H_2S in relaxation of the IAS to EFS.

Conclusion

- Contractile responses of the IAS to EFS are mediated by noradrenaline and ATP.
- Relaxation responses of the IAS are mediated mainly by NO, but H_2S and CO also contribute to the relaxation.

CHAPTER 9

9 GENERAL DISCUSSION

Stress urinary incontinence and faecal incontinence are associated with loss of tone in the circular smooth muscle of the urethra and IAS respectively. Thus, stress urinary incontinence and faecal incontinence could be treated with pharmacological agents that can increase tone of the circular smooth muscle. On the other hand, healing of anal fissures associated with hypertonia of the IAS (Farid et al., 2009; Mapel et al., 2014) may be aided by inhibition of the smooth muscle contraction.

Several pharmacological agents have been produced in the past which target the $\alpha_{1A/L}$-ARs in urethral smooth muscle (Blue et al., 2004; Knepper et al., 1995; Obika et al., 1995) and IAS (Carapeti et al., 1999a; 2000a;b; Cheetham et al., 2001). However, none of the developed agents have progressed through to a clinically useful treatment. For example the α_{1A}-AR agonist Ro 115–1240 was discontinued due to undisclosed reasons. Failure to develop clinically useful α_{1A}-AR agonists for stress urinary incontinence and anorectal disorders has been linked to cardiovascular adverse effects (Radley et al., 2001; Segev et al., 2015) and desensitisation (Akinaga et al., 2013; Perez-Aso et al., 2013). Segev et al., (2015) showed that phenylpropanolamine significantly increased systolic, diastolic and mean blood pressure following administration in dogs. Moreover, responses of the urethra after continuous administration of phenylpropanolamine in dogs decreased, with a recurrence of incontinence (Noel et al., 2010: White & Pomeroy, 1989). The development of new pharmacotherapy for these conditions will rely on in-depth knowledge of the urethral and anorectal physiology and pharmacology.

This study investigated signalling pathways involved in urethral tissues and neurotransmitters associated with normal urethral and anorectal physiology. These tissues contract to α_{1A}- AR agonists and our group has shown that contraction of these tissues is mediated by the $\alpha_{1A/L}$-AR subtype (Bagot and Chess-Williams, 2006).

The thesis was based on three hypotheses; 1) the urethral urothelium is a key modulator of the urethral tissue function. 2) The Rho kinase pathway is a key modulator of urethral tissue basal tone and contractile tone and 3) co-release of a number of neuronally-released transmitters modulate the IAS responses. The importance of the neurotransmitters and the

Rho kinase pathway in governing the basal tone and contractile responses may highlight them as a drug target for treatment of stress urinary incontinence, faecal incontinence and anal fissures.

Effect of age, region and urothelium

Since age is a risk factor for urinary incontinence (Komesu et al., 2016), in this study, the effect of age on the receptor-mediated responses in the proximal urethral of the pig was highlighted. Age did not affect the α_1-AR-mediated responses of this tissue, which was consistent with previous reports in the bladder (Kolta et al., 1984; Lluel et al., 2003) and urethra (Bagi et al., 2002), and other studies reporting the finding that age does not affect urethral responses (Ahmed et al., 2000; Suzuki et al., 1999). Thus, other contributing factors such as changes in the urothelium may be responsible for development of stress urinary incontinence. Similarly, the inhibitory effect of the urothelium/LP in this study was comparable between the young and the older porcine tissues. However, age did influence agonist-induced desensitisation of the urethra, with desensitisation of subsequent responses to phenylephrine in the older porcine urethral tissues being greater (67.2%) than those of the younger tissues (33.1%). Whether these findings reflect the human situation remains to be verified. However, if so, this may provide some insight for future drug development.

The region of the urethra responsible for greatest contractility to α_1-AR stimulation was also investigated. This study showed a regional variation in α_1-AR-mediated responses with the proximal urethral showing the greatest α_1-AR-mediated responses to agonists, which supports previous studies using pharmacological techniques (Walters et al., 2006) and cystometry/profilometry (Aagaard et al., 2014; Kirby et al., 2015), confirming the pig urethra as a suitable model for human.

To our knowledge, this study shows for the first time that the urethral urothelium has an inhibitory effect on the underlying smooth muscle response. The literature has shown this previously only in the bladder (Hawthorn et al., 2000; Templeman et al., 2002). In this study, we showed that in response to activation of α_{1A}-ARs, β-ARs and mAChRs, the underlying smooth muscle responses of the urethra were modulated by the presence of the urothelium/LP. In response to α_{1A}-AR activation, NO or prostaglandins did not mediate the inhibitory effect

of the urothelium/LP, which supports reports in the bladder (Guan et al., 2014a; Hawthorn et al., 2000). In relation to β-AR-mediated response, the urothelium/LP reduced the β-AR-mediated relaxation to isoprenaline. Although the nature of the urothelial factors in urethra was not elucidated in the present study, the findings do highlight that the role of the urothelium/LP needs to be considered in the future drug design and development and pharmacological treatments.

Urethral tone

In this study, we found that basal tone increased in isolated urethral tissues during the equilibration period, which fuelled our interest in finding factors and signalling pathways responsible for this effect. We found a significant contribution of Ca^{2+} influx via L-type Ca^{2+} channels to this basal tone, whilst Ca^{2+} release from the sarcoplasmic reticulum does not contribute to basal tone. Inhibition of cyclooxygenase also confirmed a role for prostaglandins in urethral tone maintenance, and Rho kinase, but not PKC, contributes to urethral basal tone.

In the bladder, prostaglandins are released from the urothelium in response to receptor activation (Nile et al., 2010; Nile & Gillespie, 2012), and have been shown to be involved in the regulation of detrusor smooth muscle contractility in both rodents and humans (Creed & Callahan, 1989; Klausner et al., 2011; Kobayter et al., 2012; McCafferty et al., 2008). Prostaglandins increased intracellular Ca^{2+} in guinea-pig bladder detrusor smooth muscle, and prostaglandin E_2 led to an inhibition of the spontaneous transient Ca^{2+}-activated K^+ (BK) currents in guinea pig detrusor, elevation of intracellular Ca^{2+} levels in freshly-isolated detrusor smooth muscle cells, and augmentation of detrusor smooth muscle phasic contractions (Parajuli et al., 2014). In mouse detrusor smooth muscle, prostaglandin E_2 has been reported to cause cell membrane depolarization, to increase Ca^{2+} oscillations, and to potentiate phasic contractions in the mouse bladder (Kobayter et al., 2012). A link between prostaglandin and Rho kinase has been shown in the bladder using Rho kinase inhibitors and cyclooxygenase inhibitors, which affected spontaneous rhythmic contraction amplitude following a stretch of bladders of rabbits (Komari et al., 2013). Moreover, Rho kinase mediated contractile responses following activation of prostaglandin receptors in the mesenteric artery of the rat (Kobayashi et al., 2011) and guinea-pig aorta (Shum et al., 2003).

Thus, prostaglandins may induce an increase in intracellular Ca^{2+} via the L-type Ca^{2+} channels and activate Rho kinase in the urethra, thereby mediating basal tone. Thus, prostaglandins appear to play a role in urethral basal tone, via Rho kinase activation, and this pathway may be a potential target for drug development.

Receptor-mediated responses of urethra

The greatest α_1-AR-mediated responses and potency was recorded for A61603 in the urethra tissue, compared to noradrenaline and phenylephrine. This supports reports in the literature, with A61603 referred to as a highly potent and selective α_{1A}-AR (Argyle & McGrath, 2000; Knepper et al., 1995). The greater potency of A61603 may be due to its ability to more potently stimulate phosphoinositide hydrolysis (Knepper et al., 1995). Thus, phosphoinositide hydrolysis could be targeted to pharmacologically achieve greater contraction of the urethral smooth muscle. Also, other intracellular pathways activated by A61603 may be responsible for this greater potency, and this is discussed later.

Whilst the α_{1A}-AR has been confirmed as the main receptor mediating urethral contractile responses, the underlying signalling pathways are yet known, and were investigated in the present study. At maximal contractile responses, sarcoplasmic reticulum-released Ca^{2+} contributed most to elicit responses to phenylephrine; whilst Ca^{2+} influx via L-type Ca^{2+} channels were more important for responses to noradrenaline and PKC contributed greatly to A61603-induced contractions. The contribution of Ca^{2+}-sensitisation to α_1-AR-mediated responses was greatest for A61603 for all response levels examined, suggesting a correlation between the Ca^{2+}-sensitisation pathway and higher potency and efficacy, in addition to the greater induction of phosphoinositide hydrolysis reported for A61603 by Knepper et al., (1995). Thus this result shows that the greatest response mediated by α_{1A}-AR activation is due to activation of Ca^{2+}-sensitisation/PKC pathway.

The phenomenon of desensitisation of α_1-AR-mediated responses was investigated in chapter 6 where the maintenance of urethral tissue tone after α_1-AR-activation with a single agonist concentration for 120 minutes was described. Tone maintenance was greatest for A61603-contracted tissues and lowest for noradrenaline-contracted tissues, with tone relaxing below the basal level for noradrenaline. A possible explanation for the difference in desensitisation

observed is a difference in intracellular signalling pathways and the differing contribution of Rho kinase and PKC to Ca^{2+} sensitization, as well as Ca^{2+} sources. Moreover, the urothelium and its released factors may modulate the receptor-mediated responses. However, we confirmed that the urothelium/LP does not contribute to the maintenance of urethral tone following α_1-AR activation and tone maintenance was not associated with Rho kinase or prostaglandins.

Alpha$_1$-ARs are known to be subject to phosphorylation and internalisation upon exposure to noradrenaline or phenylephrine (Vazquez-Prado et al., 2000). It is well established that α_{1A}-ARs are less phosphorylated and internalised on exposure to α_1-AR-agonists such as noradrenaline or phenylephrine than are the α_{1B}-AR and α_{1D} –AR subtypes, at least in Rat-1 fibroblasts (Vazquez-Prado et al., 2000). Since the α_{1A}-AR mediates contraction of the urethra, this may be a therapeutic advantage and means that desensitisation will be less of a problem in the urethra. However, the α_{1A}-AR still undergoes desensitisation. In chapter 6, it was also shown that prior incubation with an equipotent concentration of α_1-AR-agonist such as phenylephrine and A61603, but not noradrenaline, desensitises subsequent contractions to the physiological agonist noradrenaline. The potency of the α_1-AR-agonist was proportional to the degree of desensitisation. Such a relationship has previously been reported for β-AR agonists, where salmeterol (lower efficacy β-AR agonist) induced less desensitisation than formoterol (Moore et al., 2007; January et al., 1997; 1998) or isoprenaline (higher efficacy β-AR agonists) in HEK-293 cells and human airway smooth muscle cells respectively. Furthermore, the low efficacy agonist (salmeterol) caused less β-AR phosphorylation and internalisation than the full agonist formoterol (January et al., 1997; 1998). In addition, salmeterol showed weak efficacy for recruitment of arrestin (Moore et al., 2007), activation of adenylyl cyclase (Tran et al., 2004), and activation of GRK (Tran et al., 2004). Salmeterol did not induce significant β_2-AR internalisation or degradation in HEK-293 cells and was incapable of stimulating the translocation of arrestin (Moore et al., 2007). Thus, A61603 may be able to induce greater GRK/PKC activation than phenylephrine, which may be responsible for the greatest desensitisation by A61603.

In this study, looking at the various time points, desensitisation of α_1-ARs probably occurred within the first 15 minutes. Desensitisation and internalisation of α_1-ARs in response to α_{1A}-AR agonist has been shown in HEK-293 cells to occur at 5 minutes after exposure to agonists

(Akinaga et al., 2013). They showed that the α_{1A}-AR agonist induces GRK-dependent α_{1A}-AR phosphorylation, followed by rapid desensitisation and internalisation (~40% internalisation after 5 minutes of stimulation). Short-term exposure to this agonist could induce a long-term desensitisation of the α_{1A}-ARs.

Why noradrenaline did not induce desensitisation to subsequent contractions of the urethral tissues to noradrenaline is not known. However, a similar observation has been reported in the rat tail artery and vas deferens, where noradrenaline did not induce desensitisation (Akinaga et al., 2013). It is possible that the intracellular signalling pathway activated by noradrenaline may differ from those induced by phenylephrine and A61603.

In chapter 6 it was concluded that desensitisation due to phenylephrine is associated with changes in Rho kinase (100%), whilst that due to A61603 is associated with Ca^{2+} influx via L-type channels, Rho kinase and PKC. Another intriguing observation was that Rho kinase contributed to both phenylephrine and A61603-induced desensitisation. Rho kinase is usually associated with contraction and Ca^{2+}-sensitisation in the urethra (Teixeira et al., 2007; Walsh et al., 2011). This may suggest a feedback mechanism by which Rho kinase prevents chronic activation of receptors by regulating receptor desensitisation, as suggested by Cario-Toumaniantz et al., (2012).

A post-junctional interaction between the adrenergic and muscarinic system in the urethra was investigated in chapter 7. We showed that prior incubation with α_1-AR agonists induced desensitisation of carbachol-mediated responses, indicating a heterologous form of desensitisation involving desensitisation of unrelated receptors. Our results show that prior incubation with carbachol induced homologous desensitisation of carbachol-mediated responses in urethral smooth muscle (52.5%). However, desensitisation with carbachol of noradrenaline-induced responses in smooth muscle alone, but not in intact tissues with urothelium/LP, suggests that the urothelium/LP masks desensitisation to carbachol. This masking effect of the urothelium/LP on carbachol-induced desensitisation of noradrenaline-mediated responses was not due to neuronal release of factors or prostaglandins. The clinical application of this result is as yet unknown. For noradrenaline, a different form of desensitisation was found, with a noradrenaline mediating desensitisation of mAChR-mediated responses with no significant effect on α_{1A}-AR responses.

In conclusion, responses of the urethra are a result of interactions between the urothelium/LP and underlying smooth muscle. Moreover, the signalling pathway controlling responses is Ca^{2+} sensitization, as this pathway does not only modulate contraction but also regulates receptor activity by mediating desensitisation, which might involve the activation of kinases responsible for initiating phosphorylation, internalisation and degradation of receptor. It would be myopic or presumptive not to suggest that Rho kinase and PKC may have effects on gene expression in the urethra. Presently, the gene expression being modulated by Ca^{2+}-sensitization in the urethra is unknown, but this study foresees modulation of expression of proteins associated with contraction and desensitisation. To our knowledge, such interaction and the effects of Rho kinase/PKC on protein upregulation has not be shown in the urethra. However, in vascular smooth muscle cells of mouse and human, activated PKC and Rho kinase induced expression of contractile smooth muscle markers, which could be partially or completely repressed by inhibitors of L-type Ca^{2+} channels, PKC, and Rho kinase (Hien et al., 2016). Activated Rho kinase has also been shown to upregulate other proteins such as fibronectin in resident mesangial cells (Peng et al., 2008). Rho kinase inhibition, RhoA siRNA and dominant-negative RhoA markedly attenuated fibronectin upregulation thus confirming Rho kinase mediated upregulation of fibronectin. Thus, Ca^{2+} sensitization could upregulate proteins involve in smooth muscle contraction and desensitisation of receptor-mediated responses in the urethra.

Transmitters contributing to internal anal sphincter responses to electrical field stimulation

In the pig internal anal sphincter, guanethidine (10 μM) which is a neurotoxin selective for adrenergic nerves and abolishes adrenergic nerve transmission (Cobine et al., 2007), depressed contractions completely from a large contraction above the baseline to one that failed to increase to the baseline level following the initial relaxation response, suggesting a prolonged relaxation mechanism. Thus guanethidine inhibited contraction by > 100%. Moreover, desensitisation of P2X receptors also depressed responses to EFS indicating an excitatory neurotransmitter role for ATP in this tissue. The reduction of contraction to EFS by α,β-mATP was greater at 5 Hz stimulation than at 10Hz suggesting that ATP provides a greater contribution to contractions at lower frequencies of stimulation. This idea is

supported by the report that the inhibition of ATP breakdown has a greater potentiating effect on the neurogenic responses of visceral and vascular smooth muscle at low frequencies compared to higher frequencies (Khurana et al., 2004). Thus the present results suggest a role for ATP in the neurogenic contractions of the anal sphincter via P2X receptors, at least at lower frequencies of stimulation.

After inhibition of the contractile responses, relaxation responses to EFS were greatly enhanced. The NOS inhibitor L-NNA (100 μM) reduced the relaxation induced by field stimulation by approximately 40-60%, suggesting that NO is responsible for approximately half of the relaxation response of the sphincter to nerve stimulation. The non-nitrergic relaxation induced by EFS in the presence of Phe-Leu-VIP or indomethacin, inhibitors of VIP receptor and cyclooxygenase respectively, was not affected thus ruling out VIP or cyclooxygenase product in relaxation of the pig IAS, a conclusion also reached by Acheson et al., (2009) in the sheep IAS. However, the relaxation responses were reduced by approximately 70% in the presence of guanylate cyclase inhibitor ODQ. ODQ did have a significant effect which suggests the involvement of CO in the relaxation of this tissue since CO mediates smooth muscle relaxation responses via cGMP as shown in the opossum sphincter (Rattan et al., 2004). Using immunocytochemistry in the same animal, the enzyme heme oxygenase, which produces CO, has been found to be present in the myenteric and submucosal neurones of the IAS as well as in the interstitial cells of Cajal found in this tissue (Battish et al., 2000). ZnPPIX, an inhibitor of heme oxygenase reduced IAS relaxation responses by about 20% and this effect was not additive with ODQ. These data suggest that the ODQ sensitive component of the relaxation is composed of predominantly NO (50%) but with a smaller contribution from CO (20%).

In the presence of ODQ, the remaining small, non-NO, and non-CO relaxation was found to be H_2S, mediating its effect via the cGMP-independent pathway. Inhibition of H_2S synthesis with PAG/AOAA depressed relaxation responses to EFS and the depression was additive to that of ODQ and therefore via a cGMP-independent pathway, thus indicating a small role for H_2S in relaxation of the porcine IAS to EFS.

The neurogenic contraction of the porcine IAS can be attributed mainly to noradrenaline and ATP, with both neurotransmitters probably released from adrenergic nerves.

Neurotransmission, at least in the pig IAS, does not appear to involve ACh. The relaxations of the IAS to EFS appears to be mediated by the simultaneous release of all three gasotransmitters with relative contributions nitric oxide > carbon monoxide >H_2S. Non-adrenergic non-cholinergic relaxations of the IAS have been shown in other species, with an involvement of nitric oxide, carbon monoxide, and possibly H_2S. However, this study demonstrates the simultaneous release of all three gasoneurotransmitters, and their combined contributions, in mediating relaxation of the porcine IAS. All these transmitters represent possible targets for drug development where enhancing sphincter tone may aid the treatment of faecal incontinence or reductions in IAS tone can aid healing of anal fissures.

Final Conclusion

Ca^{2+} sensitization is a significant pathway involved in the mediation of urethral contractile responses and basal tone, as well as modulation of α_{1A}-adrenoceptor desensitisation. Thus, the Ca^{2+} sensitization pathway stands out as a prospective drug target for the development of new treatments for lower urinary tract symptoms such as stress urinary incontinence.

Further research is necessary to determine the relative contribution of the Ca^{2+} sensitization pathways in incontinence, as there is the possibility of a switch towards a reduced Ca^{2+} sensitization mechanism, which could underlie stress urinary incontinence and faecal incontinence, whilst greater Ca^{2+} sensitization may underlie hypertonia of the internal anal sphincter and inhibition of healing of anal fissures.

10 REFERENCES

Aagaard, M., Klarskov N., et al. (2014). "Urethral pressure reflectometry, a novel technique for simultaneous recording of pressure and cross-sectional area in the prostatic urethra: testing in men without bothersome lower urinary tract symptoms." Scandinavian Journal of Urology **48**(2): 195-202.

Abbracchio, M.P., Burnstock G., et al. (2006). "International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy." Pharmacological reviews **58**(3): 281-341.

Abdel-Rahman, A. A., & Carroll, R. G. (2007). "Structure and Function of the Lower Urinary Tract." xPharm: The Comprehensive Pharmacology Reference (1-2).

Abrams, P., Cardozo L. & Fall M. (2002). "The standardisation of terminology of lower urinary tract function: report from the standardisation sub-committee of the International Continence Society." Neurourology and Urodynamics **21**:167–178.

Abrams, P., Chapple C., et al. (2013). "Evaluation and treatment of lower urinary tract symptoms in older men." Journal of Urology **189**(1 Suppl): S93-S101.

Acheson, A., Rayment, S., et al. (2009). "Investigation of the role of adrenergic and non-nitrergic, non-adrenergic neurotransmission in the sheep isolated internal anal sphincter." Neurogastroenterology and Motility **21**(3): 335-345.

Achtstatter, T., Moll R., et al. (1985). "Cytokeratin polypeptide patterns of different epithelia of the human male urogenital tract: immunofluorescence and gel electrophoretic studies." Journal of Histochemistry and Cytochemistry **33**(5): 415-426.

Adie, E.J. & Milligan G. (1994). "Agonist regulation of cellular Gs alpha-subunit levels in neuroblastoma x glioma hybrid NG108-15 cells transfected to express different levels of the human beta 2 adrenoceptor." Biochemical Journal **300** (Pt 3): 709-715.

Afeli, S.A., Rovner E.S. & Petkov G.V. (2013). "BRL37344, a ß3-adrenergic receptor agonist, decreases nerve-evoked contractions in human detrusor smooth muscle isolated strips: role of BK channels." Urology **82**(3): 744.e1-e7.

Agartan, C.A., Whitbeck C., et al. (2005). "Effect of age on rabbit bladder function and structure following partial outlet obstruction." Journal of Urology **173**(4): 1400-1405.

Aguero, J., Almenar L., et al. (2012). "Myocardial G protein receptor-coupled kinase expression correlates with functional parameters and clinical severity in advanced heart failure." Journal of Cardiac failure **18**(1): 53-61.

Ahlquist, R. P. (1948). "A study of the adrenotropic receptors." American Journal of Physiology **153**(3), 586-600.

Ahmed, H., Moriyama, N., et al. (2000). "Contractile properties of urethral smooth muscles of young and aged female dogs: Morphological and pharmacological aspects." International Journal of Urology **7**(8), 298-306.

Ahmed, E.A. & Youssif M.E. (2009). "Immunohistochemical study of c-KIT (CD117) expression in renal cell carcinoma." Journal of the Egyptian National Cancer Institute **21**(2): 121-32.

Aizawa, N., Igawa Y., et al. (2010). "Effects of CL316, 243 a ß3-adrenoceptor agonist, and intravesical prostaglandin E2 on the primary bladder afferent activity of the rat." Neurourology and Urodynamics **29**: 771–776.

Akinaga, J., Lima V., et al. (2013). "Differential phosphorylation, desensitization, and internalization of a1A-adrenoceptors activated by norepinephrine and oxymetazoline." Molecular Pharmacology **83**(4): 870-81.

Alan, C., Eren A.E., et al. (2015). "Efficacy of Duloxetine in the Early Management of Urinary Continence after Radical Prostatectomy." Current Urology **8**(1):43-48.

Albano, G.D., Zhao J., et al. (2015). "IL-13 desensitizes ß2-adrenergic receptors in human airway epithelial cells through a 15-lipoxygenase/G protein receptor kinase 2 mechanism." Journal of Allergy and Clinical Immunology **135**(5): 1144-53.e1-e9.

Alexander, S.P., Mathie A. & Peters J.A. (2011). "Guide to Receptors and Channels (GRAC), 5th edition." British Journal of Pharmacology **164** (Suppl 1): S1-S324.

Alexandre, E.C., Kiguti L.R., et al. (2016). "Mirabegron relaxes urethral smooth muscle by a dual mechanism involving ß3 -adrenoceptor activation and a1 -adrenoceptor blockade." British Journal of Pharmacology **173**(3): 415-428.

Al-Noah, Z., McKenna D., et al. (2014). "Nitrergic relaxations and phenylephrine contractions are not compromised in isolated urethra in a rat model of diabetes." Autonomic Neuroscience; basic and clinical **183**: 58-65.

Alsadius, D., Hedelin M., et al. (2012). "Mean absorbed dose to the anal-sphincter region and fecal leakage among irradiated prostate cancer survivors." International journal of Radiation Oncology, biology, Physics **84**(2): e181-e185.

Alvarez, S.M., Miner A.S., et al. (2010). "Failure of Bay K 8644 to induce RhoA kinase-dependent calcium sensitization in rabbit blood vessels." British Journal of Pharmacology **160**(6): 1326-1337.

Ameda, K., Sullivan M.P., et al. (1999). "Urodynamic characterization of nonobstructive voiding dysfunction in symptomatic elderly men." Journal of Urology **162**(1): 142-146.

An, J.Y., Yun H.S., et al. (2002). "The intracellular pathway of the acetylcholine-induced contraction in cat detrusor muscle cells." British Journal of Pharmacology **137**: 1001–1010.

Andersson, K. E. (2001). "Neurotransmission and drug effects in urethral smooth muscle." Scandinavian Journal of Urology and Nephrology **35**(207): 26-34.

Andersson, K. E. (2007). "LUTS treatment: Future treatment options." Neurourology and Urodynamics **26**(6): 934-947.

Andersson, K. E., & Arner, A. (2004). "Urinary bladder contraction and relaxation: physiology and pathophysiology." Physiological Reviews **84**(3): 935-986.

Andersson, K.E, Ek A. & Persson C.G. (1977). "Effects of prostaglandins on the isolated human bladder and urethra." Acta Physiological Scandinavica **100**(2):165-171.

Andersson, K. E., Larsson, B., & Sjogren, C. (1984). "Characterization of the alpha-adrenoceptors in the female rabbit urethra." British Journal of Pharmacology **81**(2): 293-300.

Andersson, M.C., Tobin G. & Giglio D. (2008). "Cholinergic nitric oxide release from the urinary bladder mucosa in cyclophosphamide-induced cystitis of the anaesthetized rat." British Journal of Pharmacology **153**(7): 1438-1444.

Andersson, M., Aronsson P., et al. (2012). "Muscarinic receptor subtypes involved in urothelium-derived relaxatory effects in the inflamed rat urinary bladder." Autonomic neurosciences **170**(1-2): 5-11.

Antropoli, C., Perrotti P., et al. (1999). Nifedipine for local use in conservative treatment of anal fissures: preliminary results of a multicenter study. Diseases of the Colon and Rectum **42**(8): 1011–1015.

Apodaca, G., Balestreire, E., & Birder, L. A. (2007). "The uroepithelial-associated sensory web." Kidney International **72**(9): 1057-1064.

Argyle, S. A., & McGrath, J. C. (2000). "An alpha (1A)/alpha (1L)-adrenoceptor mediates contraction of canine subcutaneous resistance arteries." Journal of Pharmacology and Experimental Therapeutics **295**(2): 627-633.

Asano, T., Suzuki, T., et al. (1989). "Vasodilator actions of HA1077 in vitro and in vivo putatively mediated by the inhibition of protein kinase." British Journal of Pharmacology **98**(4): 1091-1100.

Ashworth, P.D., & hagan M.P (1993). "Some social consequences of non-compliance with pelvic floor excercise." Physiotherapy **79**: 465-471.

Avelino, A., Cruz C., et al. (2002). "Vanilloid receptor 1 expression in the rat urinary tract." Neuroscience **109**(4): 787-798.

Aydin, A., Ucak R., et al. (2002). "Vascular plexus is a differentation criterion for muscularis mucosa from muscularis propria in small biopsies and transurethral resection materials of urinary bladder?" International Urology and Nephrology **34**(3):315-319.

Ayeleke, R.O., Hay-Smith E.J & Omar M.I. (2015). "Pelvic floor muscle training added to another active treatment versus the same active treatment alone for urinary incontinence in women." Cochrane Database Systematic Reviews **11**: CD010551.

Badra, S, Andersson KE, et al. (2013). "Long-term structural and functional effects of autologous muscle precursor cell therapy in a nonhuman primate model of urinary sphincter deficiency." Journal Urology **190**(5): 1938–1945.

Badvie, S. & Andreyev H.J. (2005). "Topical phenylephrine in the treatment of radiation-induced faecal incontinence." Clinical Oncolology **17**(2): 122–126.

Baek, I., Jeon S.B., et al. (2009). "A role for Rho-kinase in Ca-independent contractions induced by phorbol-12,13-dibutyrate." Clinical and Experimental Pharmacology and Physiology **36**(3): 256-261.

Bagi, P. (2002). "Pressure/cross-sectional area relations in the proximal urethra of healthy males. Part III: the time dependent pressure response following forced dilation: standardization of a technique." Urology Research **30**(1): 1-8.

Bagi, P., Botker, R., & Kristensen, J. K. (2002). "Pressure /cross-sectional area relations in the proximal urethra of healthy males: the time dependent pressure response following forced dilation. Part IV: results in healthy volunteers." Urology Research **30**(1): 9-14.

Bagot, K., & Chess-Williams, R. (2006). Alpha1A/L-adrenoceptors mediate contraction of the circular smooth muscle of the pig urethra. Autonomic and Autacoid Pharmacology **26**(4), 345-353.

Bai, Y., Lu H. & Machida C.A. (2006). "CRM 1-mediated degradation and agonist-induced down-regulation of beta-adrenergic receptor mRNAs." Biochimica et biophysica acta **1763**(10): 1076-1089.

Bailey, H.R., Beck D.E., et al. (2002). "A study to determine the nitroglycerin ointment dose and dosing interval that best promote the healing of chronic anal fissures." Disease of the Colon and Rectum **45**(9): 1192-1199.

Bailey, K.L., Bonasera S.J., et al. (2014). "Aging causes a slowing in ciliary beat frequency, mediated by PKCe." American Journal of Physiology; Lung Cellular and Molecular Physiology **306**(6): L584-L589.

Balijepalli, R.C., Foell J.D., et al. (2006). "Localization of cardiac L-type Ca(2+) channels to a caveolar macromolecular signaling complex is required for beta(2)-adrenergic regulation." Proceedings of the national academy of sciences of the United States of America **103**(19):7500-7505.

Ballester, C., Sarriá B., et al. (2010). "Relaxation by beta 3-adrenoceptor agonists of the isolated human internal anal sphincter." Life sciences **86**(9-10): 358-364.

Banwait, K.S. and Rattan S. (2003). "Role of nitric oxide in ß3-adrenoceptor activation on basal tone of internal anal sphincter." American Journal of Physiology; Gastrointestinal and Liver Physiology **285**(3): G547-G555.

Barleben, A., & Mills, S. (2010). "Anorectal Anatomy and Physiology." Surgical Clinics of North America **90**(1): 1-15.

Bartolo, D.C., Roe A.M., et al. (1985). Evacuation proctography in obstructed defaecation and rectal intussusception. British Journal of Surgery **72** Suppl: S111–S116.

Bastan, R., Eskandari N., et al. (2014). "Role of protein phosphatases inhibitors on the histamine release and the functional desensitization in human lung mast cells." Journal of Research in Medical Sciences **19**(10): 923-927.

Battaini, F. & Pascale A. (2005). "Protein kinase C signal transduction regulation in physiological and pathological aging." Annals of the New York Academy of Sciences **1057**:177-192.

Battish, R., Cao G.Y., et al. (2000). "Heme oxygenase-2 distribution in anorectum: colocalization with neuronal nitric oxide synthase." American Journal of Physiology; Gastrointestinal and Liver Physiology **278**(1): G148-G155.

Beaty, J.S. & Shashidharan M. (2016). "Anal fissure." Clinics in colon and rectal surgery **29**(1): 30-37.

Beckel, J.M. & Birder L.A. (2012). "Differential expression and function of nicotinic acetylcholine receptors in the urinary bladder epithelium of the rat." The Journal of Physiology. **590**(6): 1465–1480.

Beckel, J.M., Daugherty S.L., et al. (2015). "Pannexin 1 channels mediate the release of ATP into the lumen of the rat urinary bladder." The Journal of Physiology **593** (8): 1857-1871.

Beckel, J.M., Kanai A., et al. (2006). "Expression of functional nicotinic acetylcholine receptors in rat urinary bladder epithelial cells." American Journal of Physiology; Renal Physiology **290**(1): F103-F110.

Bell, D., Duffin A., et al. (2014a). "A double-blind, placebo-controlled, randomised, parallel-group, dose-escalating, repeat dose study in healthy volunteers to evaluate the safety, tolerability, pharmacodynamic effects and pharmacokinetics of the once daily rectal application of NRL001 suppositories for 14 days." Colorectal Diseases **16** (Suppl 1):36-50.

Bell, D., Duffin A., et al. (2014b). "A randomised, controlled, crossover study to investigate the pharmacodynamics, pharmacokinetics and safety of 1R,2S-methoxamine hydrochloride (NRL001) in healthy elderly subjects." Colorectal Diseases **16** (Suppl 1): 27-35.

Belvisi, M.G., Patel H.J., et al. (1996). “Paradoxical facilitation of acetylcholine release from parasympathetic nerves innervating guinea-pig trachea by isoprenaline.” British Journal of Pharmacology **117**(7): 1413-1420.

Benarroch, E.E. (2007). “Enteric nervous system: functional organization and neurologic implications.” Neurourology **69**(20): 1953-1957.

Benes, J., Varejkova E., et al. (2012). “Decrease in heart adrenoceptor gene expression and receptor number as compensatory tool for preserved heart function and biological rhythm in M(2) KO animals.” Naunyn-Schmiedeberg's archives of Pharmacology **385**(12): 1161-1173.

Bengtsson, T., Redegren K., et al. (1996). “Down-regulation of beta3 adrenoreceptor gene expression in brown fat cells is transient and recovery is dependent upon a short-lived protein factor.” Journal of Biological Chemistry **271**(52): 33366-33375.

Benovic, J.L, Staniszewski C., et al. (1988). "beta-Adrenergic receptor kinase. Activity of partial agonists for stimulation of adenylate cyclase correlates with ability to promote receptor phosphorylation." Journal of Biological Chemistry **263**(8):3893-3897.

Berghmans, L.C., Hendriks H.J. et al. (1998). Conservative treatment of stress urinary incontinence in women: a systematic review of randomized clinical trials. British Journal of Urology **82**(2): 181-191.

Bernabe, J., Julia-Guilloteau V., et al. (2008). “Peripheral neural lesion-induced stress urinary incontinence in anaesthetized female cats.” British Journal of Urology international **102**(9): 1162-1167.

Berndt, G., Grosser N. et al. (2004). A common pathway of nitric oxide release from AZD3582 and glyceryl trinitrate. European Journal of Pharmaceutical Sciences **21**(2-3):331-335.

Bharucha, A.E. (2006). “Pelvic floor: anatomy and function.” Neurogastroentorology and Motility **18**(7): 507-519.

Bharucha, A.E., Dunivan G., et al. (2015). “Epidemiology, pathophysiology, and classification of fecal incontinence: state of the science summary for the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) workshop.” American Journal of Gastroentorology **110**(1): 127-136.

Biancani, P., Walsh J. & Behar J. (1985). “Vasoactive intestinal peptide: a neurotransmitter for relaxation of the rabbit internal anal sphincter.” Gastroenetrology **89**(4): 867-874.

Bicer, H., Korgali E., et al. (2012). “Contractile responses of urinary bladder in an experimental model of chronic renal failure.” Bosnian Journal of Basic Medical Sciences **12**(4): 219-223.

Biers, S.M., Reynard J.M. et al. (2006). The functional effects of a c-kit tyrosine inhibitor on guinea-pig and human detrusor. British Journal of Urology International **97**(3): 612-616.

Birder, L. A. (2010). "Urothelial signaling." Autonomic Neuroscience **153** (1-2): 33-40.

Birder, L. A. (2011). "Urothelial signaling." Handbook of Experimental Pharmacology **202**: 207-231.

Birder, L. & Andersson K.E. (2013). "Urothelial signaling." Physiology Reviews **93**(2): 653-680.

Birder, L.A., Apodaca G., et al. (1998). "Adrenergic- and capsaicin-evoked nitric oxide release from urothelium and afferent nerves in urinary bladder." American Journal of Physiology **275**: F226–F229.

Birder, L.A., Kanai A.J., et al. (2001). "Vanilloid receptor expression suggests a sensory role for urinary bladder epithelial cells." Proceeding of national academy of Science of the united states of America **98**(23): 13396-11401.

Birder, L.A., Nakamura Y., et al. (2002a). "Altered urinary bladder function in mice lacking the vanilloid receptor TRPV1." Nature Neuroscience **5**(9): 856-860.

Birder, L.A., Nealen M.L., et al. (2002b). "Beta-adrenoceptor agonists stimulate endothelial nitric oxide synthase in rat urinary bladder urothelial cells." Journal of Neuroscience **22**(18): 8063-8070.

Birder, L.A., Ruan H.Z., et al. (2004). "Alterations in P2X and P2Y purinergic receptor expression in urinary bladder from normal cats and cats with interstitial cystitis." American Journal of Physiology; Renal Physiology **287**(5): F1084-F1091.

Birder, L.A., Wolf-Johnston A., et al. (2007). Role of urothelial nerve growth factor in human bladder function. Neurourology and Urodynamics **26**(3); 405–409.

Blaivas, J.G. (1982). "The neurophysiology of micturition; a clinical study of 550 patients." Journal of Urology **127**(5): 958-963.

Blue, D. R., Daniels D. V., et al. (2004). "Pharmacological characteristics of Ro 115-1240, a selective alpha1A/1L-adrenoceptor partial agonist: a potential therapy for stress urinary incontinence." British Journal of Urology International **93**(1): 162-170.

Bogner, H.R., Gallo J.J., et al. (2002). "Anxiety disorders and disability secondary to urinary incontinence among adults over age 50." International Journal of Psychiatry in Medicine **32** (2): 141-154.

Bolego, C., Pinna C., et al. (1995). "The biphasic response of rat vesical smooth muscle to ATP." British Journal of Pharmacology **114**(8): 1557-1562.

Boopathi, E., Gomes C., et al. (2014). "Mechanical stretch upregulates proteins involved in Ca2+ sensitization in urinary bladder smooth muscle hypertrophy." American Journal of Physiology; cell physiology **307**(6): C542-C553.

Borgland, S.L., Connor M. et al. (2003). "Opioid agonists have different efficacy profiles for G protein activation, rapid desensitization, and endocytosis of mu-opioid receptors." Journal of Biological Chemistry **278**(21):18776-18784.

Borroto-Escuela, D.O., Correia P.A., et al. (2011). "Muscarinic receptor family interacting proteins: role in receptor function." Journal of neuroscience methods **195**(2): 161-169.

Borst, M.M., Marquetant, R., et al. (1997). "β- Blockade reduces effects of adenosine and carbachol by transregulation of inhibitory receptors and Gi proteins." American Journal of Physiology **272** (4): H1672–H1679 .

Boterman, M., Smits, S. R., et al. (2006). "Protein kinase C potentiates homologous desensitization of the β2-adrenoceptor in bovine tracheal smooth muscle." European Journal of Pharmacology **529**(1–3): 151-156.

Brading. (1987). "Physiology of bladder smooth muscle." Springer Verlag: New York. **p. 161**.

Brading, A.F. (1999). "The physiology of the mammalian urinary outflow tract." Experimental Physiology **84**(1): 215-221.

Brading, A.F. (2006). "Spontaneous activity of lower urinary tract smooth muscles: correlation between ion channels and tissue function." Journal of Physiology **570**(pt 1): 13-22.

Brading, A.F. & Williams J.H. (1990). "Contractile responses of smooth muscle strips from rat and guinea-pig urinary bladder to transmural stimulation: effects of atropine and alpha,beta-methylene ATP." British Journal of Pharmacology **99**(3):493-498.

Brading, A.F., McCoy R. & Dass N. (1999). "alpha1-adrenoceptors in urethral function." European Journal of Urology **36**(suppl 1): 74-79.

Brading, A. F., Teramoto N., et al. (2001). "Morphological and physiological characteristics of urethral circular and longitudinal smooth muscle." Scandinavian Journal of Urology and Nephrology **207**: 12-18.

Bradley, J.E., Anderson U.A., et al. (2004). "Characterization of T-type calcium current and its contribution to electrical activity in rabbit urethra." American Journal of Physiology; Cell physiology **286**(5): C1078-C1088.

Bradley, E., Hollywood M.A., et al. (2006). "Contribution of reverse Na+-Ca2+ exchange to spontaneous activity in interstitial cells of Cajal in the rabbit urethra." Journal of Physiology **574**(Pt 3): 651-661.

Brehmer, A. (2006). "Structure of enteric neurons." Advances in Anatomy, embryology and cell biology **186**: 1-91.

Brichetto, L., Song P., et al. (2003). "Modulation of cholinergic responsiveness through the [beta]-adrenoceptor signal transmission pathway in bovine trachealis." Journal of Applied Physiology **95**(2): 735-741.

Bridgewater, M., MacNeil H.F. & Brading A.F. (1993). "Regulation of tone in pig urethral smooth muscle." Journal of Urology **150**(1): 223-228.

Bridgewater, M., Davies J. R., & Brading A. F. (1995). Regional variations in the neural control of the female pig urethra." British Journal of Urology **76**(6): 730-740.

Brisinda, D., Maria G., et al. (1999). "A comparison of injections of botulinum toxin and topical nitroglycerin ointment for the treatment of chronic anal fissure." The New England Journal of Medicine **341** (2):65-69.

Brown, A.C., Sumfest J.M., et al. (1989). "Histopathology of the internal anal sphincter in chronic anal fissure." Diseases of the Colon and Rectum 32(8):680-683.

Brown, J.S., Vittinghoff E., et al. (2006). Prevalence and risk factors for urinary incontinence in women with type 2 diabetes and impaired fasting glucose: findings from the National Health and Nutrition Examination Survey (NHANES) 2001-2002. Diabetes Care **29**(6): 1307-1312.

Brown, S.M., Bentcheva-Petkova L.M., et al. (2008). "Beta-adrenergic relaxation of mouse urinary bladder smooth muscle in the absence of large-conductance Ca2+-activated K+ channel." American Journal of Physiology; Renal Physiology **295**(4): F1149-F1157.

Bruns, T.M., Gaunt R.A. & Weber D.J. (2011). "Multielectrode array recordings of bladder and perineal primary afferent activity from the sacral dorsal root ganglia." Journal of Neural Engineering **8**(5): 056010.

Bschleipfer, T., Schukowski K., et al. (2007). "Expression and distribution of cholinergic receptors in the human urothelium. Life Sciences **80**(24-25): 2303-2307.

Buckley, M.S., Washington S., et al. (1996). "Characterization and immunohistochemical localization of the glycoconjugates of the rabbit bladder mucosa." Archives of Biochemistry and Biophysics **330**(1): 163-173.

Buckley, M., Xin P., et al. (2000). "Lectin histochemical examination of rabbit bladder glycoproteins and characterization of a mucin isolated from the bladder mucosa." Archives of Biochemistry and Biophysics **375**(2): 270-277.

Buckley, B.S., Lapitan M.C.et al. (2010). "Prevalence of urinary incontinence in men, women, and children--current evidence: findings of the Fourth International Consultation on Incontinence." Urology **76**(2): 265-70.

Budd, D.C., Challiss R.A., et al. (1999). "Cross talk between m3-muscarinic and beta(2)-adrenergic receptors at the level of receptor phosphorylation and desensitization." Molecular Pharmacology **56**(4): 813-823.

Bump, R.C., Voss S., et al. (2008). "Long-term efficacy of duloxetine in women with stress urinary incontinence." British Journal of Urology International **102**(2): 214-218.

Bundey, R.A. & Nahorski S.R. (2001). "Homologous and heterologous uncoupling of muscarinic M (3) and alpha (1B) adrenoceptors to Galpha (q/11) in SH-SY5Y human neuroblastoma cells." British Journal of Pharmacology **134**(2): 257-264.

Buntzen, S., Nordgren S., et al. (1996). "The role of nitric oxide in the acetylcholine-induced relaxation of the feline internal anal sphincter, in vitro." Scandinavian Journal of Gastroenterology **31**(12): 1189-1194.

Buranakarl, C., Kijtawornrat A., et al. (2001). "Effects of bethanechol on canine urinary bladder smooth muscle function." Research in Veterinary Science **71**(3): 175-181.

Burdzinska, A., Crayton R., et al. (2012). "Urethral distension as a novel method to simulate sphincter insufficiency in the porcine animal model." International Journal of Urology **19**(7): 676-682.

Burleigh, D.E, D'Mello A. & Parks A.G. (1979). "Responses of isolated human internal anal sphincter to drugs and electrical field stimulation." Gastroenterology **77**: 484-490.

Burns, A.J., Lomax A.E., et al. (1996). "Interstitial cells of Cajal mediate inhibitory neurotransmission in the stomach." Proceedings of the national academy of sciences of the United States of America **93**(21): 12008-12013.

Buzelin, J.M., Fonteyne E., et al. (1997). Comparison of tamsulosin with alfuzosin in the treatment of patients with lower urinary tract symptoms suggestive of bladder outlet obstruction (symptomatic benign prostatic hyperplasia). The European Tamsulosin Study Group. British Journal of Urology **80**(4): 597-605.

Bylund, D. (1992). "Subtypes of a1 and a2-adrenergic receptors." FASEB Journals **6**: 832–839.

Bylund, D.B., Blaxall H.S., et al. (1992). "Pharmacological characteristics of alpha 2-adrenergic receptors: comparison of pharmacologically defined subtypes with subtypes identified by molecular cloning." Molecular Pharmacology **42**(1): 1-5.

Bytzer, P., Talley N.J., et al. (2001). Prevalence of gastrointestinal symptoms associated with diabetes mellitus: a population-based survey of 15,000 adults. Archives of Internal Medicine **161**(16): 1989-1996.

Cabrera-Wrooman, A., Romero-Ávila M.T. & García-Sáinz J.A. (2010). "Roles of the a1A-adrenergic receptor carboxyl tail in protein kinase C-induced phosphorylation and desensitization." Naunyn-Schmiedeberg's archives of pharmacology **382**(5-6): 499-510.

Caldwell, P.H., Sureshkumar P. & Wong W.C. (2016). "Tricyclic and related drugs for nocturnal enuresis in children." Cochrane database of Systematic Reviews **1**: CD002117.

Cameron, A.P., Jimbo M. & Heidelbaugh J.J. (2013). "Diagnosis and office-based treatment of urinary incontinence in adults. Part two: treatment." Therapeutic advances in Urology **5**(4): 189-200.

Camilleri, M., Kim D.Y., et al. (2003). "A randomized, controlled exploratory study of clonidine in diarrhea-predominant irritable bowel syndrome." Clinical Gastroenterology and Hepatology **1**(2): 111-121.

Canda, A. E., Cinar M. G., et al. (2008). "Pharmacologic targets on the female urethra." Urologia Internationalis **80**(4): 341-354.

Cannon, T. W., & Chancellor, M. B. (2003). "Pharmacotherapy for stress urinary incontinence." Reviews in urology **5**(3): 135-141.

Carapeti, E.A., Kamm M.A., et al. (1999a). "Topical phenylephrine increases anal sphincter resting pressure." British Journal of Surgery **86**(2): 267-270.

Carapeti, E.A., Kamm M.A., et al. (1999b). "Randomised controlled trial shows that glyceryl trinitrate heals anal fissures, higher doses are not more effective, and there is a high recurrence rate." Gut **44**(5): 727-730.

Carapeti, E.A., Kamm M.A. & Phillips R.K. (2000a). "Randomized controlled trial of topical phenylephrine in the treatment of faecal incontinence." British Journal of Surgery **87**(1): 38-42.

Carapeti, E.A., Kamm M.A., et al. (2000b). "Randomized, controlled trial of topical phenylephrine for fecal incontinence in patients after ileoanal pouch construction." Diseases of the colon and rectum **43**(8): 1059-1063.

Cardozo, L., Drutz H.P., et al. (2004). "Pharmacological treatment of women awaiting surgery for stress urinary incontinence." Obstetrics and Gynecology **104**(3): 511-519.

Cario-Toumaniantz, C., Ferland-McCollough D., et al. (2012). "RhoA guanine exchange factor expression profile in arteries: evidence for a Rho kinase-dependent negative feedback in angiotensin II-dependent hypertension." American Journal of Physiology; Cell Physiology **302**(9): C1394-1404.

Carlile, A., Davies I., et al. (1988). "Age changes in the human female urethra: a morphometric study." Journal of Urology **139**(3): 532-535.

Carlstedt A., Fasth S., et al. (1988). "The sympathetic innervation of the internal anal sphincter and rectum in the cat." Acta Physiologica Scandinavica **133**(3):423-431.

Carter, A.A. & Hill S.J. (2005). "Characterization of isoprenaline- and salmeterol-stimulated interactions between beta2-adrenoceptors and beta-arrestin 2 using beta-galactosidase complementation in C2C12 cells." Journal of Pharmacology and experimental therapeutics **315**(2): 839-848.

Carvajal, J. A., Germain A. M., et al. (2000). "Molecular mechanism of cGMP-mediated smooth muscle relaxation." Journal of Cell Physiology **184**(3): 409-420.

Casas-González, P. & García-Sáinz J.A. (2006). "Role of epidermal growth factor receptor transactivation in alpha1B-adrenoceptor phosphorylation." European Journal of Pharmacology **542**(1-3): 31-36.

Casas-González, P., Ruiz-Martínez A. & García-Sáinz JA. (2003). "Lysophosphatidic acid induces alpha1B-adrenergic receptor phosphorylation through G beta gamma, phosphoinositide 3-kinase, protein kinase C and epidermal growth factor receptor transactivation." Biochima et biophysica acta **1633**(2): 75-83.

Casas-González, P., Vázquez-Prado J. & García-Sáinz J.A. (2000). "Lysophosphatidic acid modulates alpha (1b)-adrenoceptor phosphorylation and function: roles of Gi and phosphoinositide 3-kinase." Molecular Pharmacology **57**(5): 1027-1033.

Castillo-Badillo, J.A., Molina-Muñoz T., et al. (2012). "Sphingosine 1-phosphate-mediated a1B-adrenoceptor desensitization and phosphorylation. Direct and paracrine/autocrine actions." Biochimica et Biophysica acta **1823**(2): 245-254.

Castillo-Badillo, J.A., Sánchez-Reyes O.B., et al. (2015). "a1B-adrenergic receptors differentially associate with Rab proteins during homologous and heterologous desensitization." Plos One **10**(3): e0121165.

Cernecka, H., Kersten K., et al. (2015). "ß3-Adrenoceptor-mediated relaxation of rat and human urinary bladder: roles of BKCa channels and Rho kinase." Naunyn-Schmiedeberg's Archives of Pharmacology **388**(7): 749-759.

Cha, S., Singson E., et al. (2006). "Muscarinic acetylcholine type-3 receptor desensitization due to chronic exposure to Sjögren's syndrome-associated autoantibodies." The Journal of Rheumatology **33**(2): 296-306.

Chacko, S., Conti M.A. & Adelstein R.S. (1977). "Effect of phosphorylation of smooth muscle myosin on actin activation and Ca^{2+} regulation." Proceedings of the National Academy of Science of the United States of America **74**(1): 129-133.

Chaiyaprasithi, B., Mang C. F., et al. (2003). "Inhibition of Human Detrusor Contraction by a Urothelium Derived Factor." Journal of Urology **170**(5): 1897-1900.

Chakder, S., Sarma D.N. & Rattan S. (2001). "Mechanism of internal anal sphincter smooth muscle relaxation by phorbol 12,13-dibutyrate." American Journal of Physiology; Gastrointestinal and Liver Physiology **280**(6): G1341-G1350.

Chaliha, C., Kalia V., et al. (1999). "Antenatal prediction of postpartum urinary and fecal incontinece." Obstetrics and Gynecology **94**(5): 689-694.

Chalothorn, D., McCune D.F., et al. (2002). "Differences in the cellular localization and agonist-mediated internalization properties of the alpha (1)-adrenoceptor subtypes." Molecular Pharmacology **61**(5): 1008-1016.

Chang, H.Y., Cheng C.L., et al. (2006). "Reflexes evoked by electrical stimulation of afferent axons in the pudendal nerve under empty and distended bladder conditions in urethane-anesthetized rats." Journal of Neuroscience methods **150**(1): 80-89.

Chapple, CR., Amarenco G., et al. (2013a). "A proof-of-concept study: mirabegron, a new therapy for overactive bladder." Neurourology and urodynamics **32**(8): 1116-1122.

Chapple, CR., Dvorak V., et al. (2013b). "A phase II dose-ranging study of mirabegron in patients with overactive bladder." International Urogynecology Journal **24**(9): 1447-1458.

Chapple, CR., Kaplan S.A., et al. (2013c). "Randomized double-blind, active-controlled phase 3 study to assess 12-month safety and efficacy of mirabegron, a ß (3)-adrenoceptor agonist, in overactive bladder." European Urology 63(2): 296-305.

Chapple, C.R., Cardozo L., et al. (2014a). "Mirabegron in overactive bladder: a review of efficacy, safety, and tolerability." Neurourology and urodynamics **33**(1): 17-30.

Chapple, C., Schneider T., et al. (2014b). "Superiority of fesoterodine 8 mg vs 4 mg in reducing urgency urinary incontinence episodes in patients with overactive bladder: results of the randomised, double-blind, placebo-controlled EIGHT trial." British Journal of Urology **114**(3): 418-426.

Charles, A.C., Mostovskaya N., et al. (2003). "Coexpression of delta-opioid receptors with micro receptors in GH3 cells changes the functional response to micro agonists from inhibitory to excitatory." Molecular Pharmacology **63**(1): 89-95.

Cheetham, M. J., Kamm M. A., & Phillips, R. K. S. (2001). "Topical phenylephrine increases anal canal resting pressure in patients with faecal incontinence." Gut **48**(3): 356-359.

Chen, C. Q., Xin, H., & Zhu, Y. Z. (2007). "Hydrogen sulfide: third gaseous transmitter, but with great pharmacological potential." Acta Pharmacologica Sinica **28**(11): 1709-1716.

Chen, C.P., Chen X., et al. (2015). "In vivo roles for myosin phosphatase targeting subunit-1 phosphorylation sites T694 and T852 in bladder smooth muscle contraction." Journal of Physiology **593**(3): 681-700.

Chen, S.C., Grill W.M., et al. (2012). "Bilateral pudendal afferent stimulation improves bladder emptying in rats with urinary retention." British Journal of Urology International **109**(7): 1051-1058.

Chen, W., Sang J.Y., et al. (2013). "Desensitization of G-protein-coupled receptors induces vascular hypocontractility in response to norepinephrine in the mesenteric arteries of cirrhotic patients and rats." Hepatobiliary & Pancreatic Diseases International **12**(3): 295-304.

Chess-Williams, R. & Bagot J.L. (2005). "Desensitisation of pig urethral but not internal anal sphincter (IAS) smooth muscle by aplha1-adrenoceptor agonists." e Journal of the British Pharmacological Society **2005**.

Chess-Williams, R., Aston N. & Couldwell C. (1994). "Alpha 1A-adrenoceptor subtype mediates contraction of the rat urethra." Journal of Autonomic Pharmacology **14**(6): 375-381.

Chia, Y.W., Fowler C.J., et al. (1995). Prevalence of bowel dysfunction in patients with multiple sclerosis and bladder dysfunction. Journal of Neurology **242**(2): 105-108.

Chiarelli, P., Bower W., et al. (2005). "Estimating the prevalence of urinary and faecal incontinence in Australia: systematic review." Australasian Journal on Ageing **24**(1): 19-19.

Chong, L.K., Suvarna K., et al. (2003). "Desensitization of beta2-adrenoceptor-mediated responses by short-acting beta2-adrenoceptor agonists in human lung mast cells." British Journal of Pharmacology **138**(3): 512-520.

Chong, E.C., Khan A.A., et al. (2011). "The financial burden of stress urinary incontinence among women in the United States." Current Urology Reports **12**(5): 358-362.

Chopra, B., Gever J., et al. (2008). "Expression and function of rat urothelial P2Y receptors." American Journal of Physiology; Renal Physiology **294**(4): F821-F829.

Chrysos, E., Xynos E., et al. (1996). Effect of nifedipine on rectoanal motility. Diseases of the Colon and Rectum **39**(2): 212–216.

Chuang, S.M., Liu K.M., et al. (2013). "Dual involvements of cyclooxygenase and nitric oxide synthase expressions in ketamine-induced ulcerative cystitis in rat bladder." Neurourology and Urodynamics **32**(8): 1137-1143.

Chuang, T.T., Paolucci L. & De Blasi A. (1996). "Inhibition of G protein-coupled receptor kinase subtypes by Ca^{2+}/calmodulin." Journal of Biological Chemistry **271**(45): 28691-28696.

Chuang, T. T., LeVine H., 3rd, & De Blasi, A. (1995). "Phosphorylation and activation of beta-adrenergic receptor kinase by protein kinase C." Journal of Biological Chemistry **270**(31): 18660-18665.

Clelland, L.J., Browne B.M., et al. (2011). "Rho-kinase inhibition attenuates calcium-induced contraction in ß-escin but not Triton X-100 permeabilized rabbit femoral artery." Journal of Research and Cell Motility **32**(2): 77-88.

Cobine, C.A., Fong M., et al. (2007). "Species dependent differences in the actions of sympathetic nerves and noradrenaline in the internal anal sphincter." Neurogastroenterology and motility **19**(11): 937-945.

Cobine, C.A., Hennig G.W., et al. (2010). Interstitial cells of Cajal in the cynomolgus monkey rectoanal region and their relationship to sympathetic and nitrergic nerves." American Journal of Physiology; Gastrointestinal and Liver Physiology **298**(5): G643-G656.

Cobine, C.A., Hennig G.W., et al. (2011). "Relationship between interstitial cells of Cajal, fibroblast-like cells and inhibitory motor nerves in the internal anal sphincter. Cell and Tissues Research **344**(1): 17-30.

Cobine, C.A., Sotherton A.G., et al. (2014). "Nitrergic neuromuscular transmission in the mouse internal anal sphincter is accomplished by multiple pathways and postjunctional effector cells." American Journal of Physiology; Gastrointestinal and Liver Physiology **307**(11): G1057-G1072.

Cockayne, D.A., Hamilton S.G., et al. (2000). "Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice." Nature **407**(6807): 1011-1015.

Cockayne, D.A., Dunn P.M., et al. (2005). "P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor subunit in mediating multiple sensory effects of ATP." Journal of Physiology **567**(Pt 2): 621-639.

Cody, J.D., Jacobs M.L, et al. (2012). "Oestrogen therapy for urinary incontinence in post-menopausal women." Cochrane Database of Systematic Reviews **10**: CD001405.

Collier, M.L., Ji G., et al. (2000). "Calcium-induced calcium release in smooth muscle: loose coupling between the action potential and calcium release." The Journal of General physiology **115**(5): 653-62.

Collste, L. & Lindskog M. (1987). "Phenylpropanolamine in treatment of female stress urinary incontinence. Double-blind placebo controlled study in 24 patients." Urology **30**(4): 398-403.

Condemi, J.J. (2001). "Comparison of the efficacy of formoterol and salmeterol in patients with reversible obstructive airway disease: a multicenter, randomized, open-label trial." Clinical therapeutics **23**(9): 1529-1541.

Conlon, K., Christy C., et al. (2009). "Pharmacological properties of 2-((R-5-chloro-4-methoxymethylindan-1-yl)-1H-imidazole (PF-3774076), a novel and selective alpha1A-adrenergic partial agonist, in in vitro and in vivo models of urethral function." Journal of Pharmacology and Experimental Therapeutics **330**(3): 892-901.

Conner, D.A., Mathier M.A., et al. (1997). "beta-Arrestin1 knockout mice appear normal but demonstrate altered cardiac responses to beta-adrenergic stimulation." Circulation Research **81**(6): 1021-1026.

Conte, B., Maggi, C. A., et al. (1991). "Simultaneous recording of vesical and urethral pressure in urethane-anesthetized rats: effect of neuromuscular blocking agents on the activity of the external urethral sphincter." Journal of Pharmacological Methods **26**(3): 161-171.

Cook, T.A, Brading A.F. & Mortensen N.J.M. (1999a). "Differences in contractile properties of anorectal smooth muscle and the effects of calcium channel blockade." British Journal of Surgery **86**(1): 70-75.

Cook, T.A, Brading A.F. & Mortensen N.J.M. (1999b). "Effects of nifedipine on anorectal smooth muscle in vitro." Diseases of the colon and rectum **42**(6): 782-787.

Cooper, P.R., Kurten R.C., et al. (2011). "Formoterol and salmeterol induce a similar degree of ß2-adrenoceptor tolerance in human small airways but via different mechanisms." British Journal of Pharmacology **163**(3): 521-532.

Copik, A.J., Ma C., et al. (2009). "Facilitatory interplay in alpha 1a and beta 2 adrenoceptor function reveals a non-Gq signaling mode: implications for diversification of intracellular signal transduction." Molecular Pharmacology **75**(3): 713-728.

Copik, A.J., Baldys A., et al. (2015). "Isoproterenol acts as a biased agonist of the alpha-1A-adrenoceptor that selectively activates the MAPK/ERK pathway." Plos One **10**(1): e0115701.

Cotecchia, S., Schwinn D.A., et al. (1988). "Molecular cloning and expression of the cDNA for the hamster alpha 1-adrenergic receptor." Proceedings of the National Academy of Sciences of the United State of America **85**(19): 7159-7163.

Coyne, K.S., Sexton C.C., et al. (2009). "The prevalence of lower urinary tract symptoms (LUTS) in the USA, the UK and Sweden: results from the Epidemiology of LUTS (EpiLUTS) study." British Journal of Urology international **104**(3): 352-360.

Creed, K.E. & Callahan S.M (1989). "Prostaglandins and neurotransmission at the guinea pig and rabbit urinary bladder." Pflugers Archives **413**(3): 299-302.

Creed, K.E., Callahan S.M. & Ito Y. (1994). "Excitatory neurotransmission in the mammalian bladder and the effects of suramin." British Journal of Urology **74**(6): 736-743.

Crema, A., Frigo G.M., et al. (1983). "Purine receptors in the guinea-pig internal anal sphincter." British Journal of Pharmacology **78**(3): 599-603.

Cribbs, L.L (2006). "T-type Ca2+ channels in vascular smooth muscle: multiple functions." Cell Calcium **40**(2): 221-230.

Crowe, R., & Burnstock G. (1989). "A histochemical and immunohistochemical study of the autonomic innervation of the lower urinary tract of the female pig. Is the pig a good model for the human bladder and urethra?" Journal of Urology **141**(2): 414-422.

Csizmadi, I., Collet J.P. et al. (2004). The effects of transdermal and oral oestrogen replacement therapy on colorectal cancer risk in postmenopausal women. British Journal of Cancer **90**(1): 76-81.

D'Agostino, G., Barbieri A., et al. (1997). "M4 muscarinic autoreceptor-mediated inhibition of -3H-acetylcholine release in the rat isolated urinary bladder." Journal of Pharmacology and Experimental Therapeutics **283**: 750–756.

D'Agostino, G., Bolognesi M.L., et al. (2000). "Prejunctional muscarinic inhibitory control of acetylcholine release in the human isolated detrusor: involvement of the M4 receptor subtype." British Journal of Pharmacology **129**(3): 493-500.

Da Silva-Santos, J.E., Chiao C.W., et al. (2009). "The Rho-A/Rho-kinase pathway is up-regulated but remains inhibited by cyclic guanosine monophosphate-dependent mechanisms during endotoxemia in small mesenteric arteries." Critical care Medicine **37**(5): 1716-1723.

Daaka, Y., Luttrell L.M., et al. (1998). "Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase." Journal of Biological Chemistry **273**(2): 685-688.

Dale, P.R., Cernecka H., et al. (2014). "The pharmacological rationale for combining muscarinic receptor antagonists and ß-adrenoceptor agonists in the treatment of airway and bladder disease." Current Opinion in Pharmacology 16: 31-42.

Daly, D.M., Chess-Williams R., et al. (2010). "The inhibitory role of acetylcholine and muscarinic receptors in bladder afferent activity." European Urology. **58**(1): 22-28; discussion 31-32.

Daly, D.M., Nocchi L., et al. (2014). "Age-related changes in afferent pathways and urothelial function in the male mouse bladder." Journal of Physiology **592**(3): 537-549.

Damon, H., Guye O., et al. (2006). "Prevalence of anal incontinence in adults and impact on quality-of-life." Gastroeneterologie clinique et biologigue **30**(1): 37-43.

Dang, V.C., Chieng B.C. & Christie M.J. (2012). "Prolonged stimulation of μ-opioid receptors produces ß-arrestin-2-mediated heterologous desensitization of α(2)-adrenoceptor function in locus ceruleus neurons." Molecular Pharmacology **82**(3): 473-480.

Daniel, E.E. & Posey-Daniel V. (1984). "Neuromuscular structures in opossum esophagus: role of interstitial cells of Cajal." American Journal of Physiology **246**(3 Pt 1): G305-G315.

Dass, N., McMurray G., et al. (2001). "Morphological aspects of the female pig bladder neck and urethra: quantitative analysis using computer assisted 3-dimensional reconstructions." Journal of Urology **165**: 1294–1299.

Dave, S., Grover V.P., et al. (2002). "The role of imipramine therapy in bladder exstrophy after bladder neck reconstruction." British Journal of Urology International **89**(6): 557-560; discussion 560-561.

Davidovskaya, T.L. & Bogutska K.I. (2003). "Modulatory effect of aluminum ions on smooth muscle contraction." Neurophysiology **4**: 314.

Davidson, R.A. & McCloskey K.D. (2005). "Morphology and localization of interstitial cells in the guinea pig bladder: structural relationships with smooth muscle and neurons." Journal of Urology **173**(4): 1385-1390.

Davies, S. P., Reddy H., et al. (2000). "Specificity and mechanism of action of some commonly used protein kinase inhibitors." Biochemical Journal **351**(Pt 1): 95-105.

De Blasi, A., Parruti G., & Sallese, M. (1995). "Regulation of G protein-coupled receptor kinase subtypes in activated T lymphocytes. Selective increase of beta-adrenergic receptor kinase 1 and 2." The Journal of Clinical Investigation **95**(1): 203-210.

De Godoy, M. A. F., Dunn S., & Rattan S. (2004). "Evidence for the role of angiotensin II biosynthesis in the rat internal anal sphincter tone. Gastroenterology **127**(1): 127-138.

De Godoy, M. A., Rattan N., & Rattan S. (2009). "COX-1 vs. COX-2 as a determinant of basal tone in the internal anal sphincter." American Journal of Physiology; Gastrointestinal and Liver Physiology **296**(2): G219-225.

De Groat, W.C (1986). "Spinal cord projections and neuropeptides in visceral afferent neurons." Progress in brain research **67**: 165-187.

De Groat, W.C. & Yoshimura N. (2009). "Afferent nerve regulation of bladder function in health and disease." Handbook of experimental Pharmacology **194**: 91-138.

De Groat, W.C. & Yoshimura N. (2015). "Anatomy and physiology of the lower urinary tract." Handbook of Clinical Neurology **130**: 61-108.

De Groat, W.C., Griffiths D. & Yoshimura N. (2015). "Neural control of the lower urinary tract." Comprehensive Physiology **5**(1): 327-396.

De Laet, K., De Wachter S. & Wyndaele J.J. (2006). "Systemic oxybutynin decreases afferent activity of the pelvic nerve of the rat: new insights into the working mechanism of antimuscarinics." Neurourology and Urodynamics **25**: 156–161.

De Lorijn, F., de Jonge W.J., et al. (2005). "Interstitial cells of Cajal are involved in the afferent limb of the rectoanal inhibitory reflex." Gut **54**(8): 1107-1113.

De Luca, A., Li C.G. & Rand M.J.(1999). "Nitrergic and purinergic mechanisms and their interactions for relaxation of the rat internal anal sphincter." Journal of Autonomic pharmacology **19**(1): 29-37.

De Wachter, S. & Wyndaele J.J. (2003). "Intravesical oxybutynin: a local anesthetic effect on bladder C afferents." Journal Urology **169**: 1892–1895.

DebBurman, S.K., Ptasienski J., et al. (1996). "G protein-coupled receptor kinase GRK2 is a phospholipid-dependent enzyme that can be conditionally activated by G protein betagamma subunits." Journal of Biological Chemistry **271**(37): 22552-22562.

Deckmann, K., Filipski K., et al. (2014). "Bitter triggers acetylcholine release from polymodal urethral chemosensory cells and bladder reflexes." Proceedings of national of academy of science of the United States of America **111**(22): 8287-8292.

Del Carmen Medina L., Vázquez-Prado J. & García-Sáinz J.A. (2000). "Cross-talk between receptors with intrinsic tyrosine kinase activity and alpha1b-adrenoceptors. The Biochemical Journal **350** Pt 2: 413-419.

Delaney, K.A., Murph M.M., et al. (2002). "Transfer of M2 muscarinic acetylcholine receptors to clathrin-derived early endosomes following clathrin-independent endocytosis." Journal of Biological Chemistry **277**(36): 33439-33446.

Dellabianca, A., Sacchi M. et al. (2007). "Role of carbon monoxide in electrically induced non-adrenergic, non-cholinergic relaxations in the guinea-pig isolated whole trachea." British Journal of Pharmacology **150**(2):220-226.

Demaagd, G.A. & Davenport TC. (2012). "Management of urinary incontinence. Pharmacy and Therapeutics **37**(6): 345H-361H.

Deng, J., He P., et al. (2012). "Identification of T-type calcium channels in the interstitial cells of Cajal in rat bladder." Urology **80**(6): 1389.e1-e7.

Deng, M., Ding W., et al. (2011). "MLCK-independent phosphorylation of MLC20 and its regulation by MAP kinase pathway in human bladder smooth muscle cells." Cytoskeleton **68**(3): 139-149.

Denninger, J. W., & Marletta M. A. (1999). "Guanylate cyclase and the NO/cGMP signaling pathway." Biochimica et Biophysica Acta **1411**(2-3): 334-350.

Deplanne, V., Palea S., & Angel I. (1998). "The adrenergic, cholinergic and NANC nerve-mediated contractions of the female rabbit bladder neck and proximal, medial and distal urethra." British Journal of Pharmacology **123**(8): 1517-1524.

Dhaese, I. & Lefebvre R.A. (2009). "Myosin light chain phosphatase activation is involved in the hydrogen sulfide-induced relaxation in mouse gastric fundus." European Journal of Pharmacology **606**(1-3): 180-186.

Dhaese, I., Van Colen I. & Lefebvre R.A. (2010). "Mechanisms of action of hydrogen sulfide in relaxation of mouse distal colonic smooth muscle." European Journal of Pharmacology **628**(1-3): 179-186.

Dhein, S., von Salisch S. & Michel M.C. (2013). "Cross-regulation between cardiac muscarinic acetylcholine receptors and β-adrenoceptors: lessons for use of knock-out mice." Naunyn-Schmiedeberg's Archive of Pharmacology **386** (1): 1–3.

Dillon-Carter, O., & Chuang D. M. (1989). "Homologous desensitization of muscarinic cholinergic, histaminergic, adrenergic, and serotonergic receptors coupled to phospholipase C in cerebellar granule cells." Journal of Neurochemistry **52**(2): 598-603.

Dinenno, F.A., Jones P.P., et al. (1999). "Limb blood flow and vascular conductance are reduced with age in healthy humans: relation to elevations in sympathetic nerve activity and declines in oxygen demand." Circulation **100**(2): 164-170.

Dinenno, F.A., Tanaka H., et al. (2001). "Reductions in basal limb blood flow and vascular conductance with human ageing: role for augmented alpha-adrenergic vasoconstriction." Journal of Physiology **536**(Pt 3): 977-983.

Ditah, I., Devaki P., et al. (2014). "Prevalence, trends, and risk factors for fecal incontinence in United States adults, 2005-2010." Clinical gastroenterology and Hepatology **12**(4): 636-643.e1-2.

Diviani, D., Lattion A.L., et al. (1996). "Effect of different G protein-coupled receptor kinases on phosphorylation and desensitization of the alpha1B-adrenergic receptor." Journal of Biological Chemistry **271**(9): 5049-5058.

Diviani, D., Lattion A. L., & Cotecchia S. (1997). "Characterization of the phosphorylation sites involved in G protein- coupled receptor kinase- and protein kinase C-mediated desensitization of the α(1B)-adrenergic receptor." Journal of Biological Chemistry **272**(45): 28712-28719.

Dixon, J.S. & Gosling J.A. (1983). "Histology and fine structure of the muscularis mucosae of the human urinary bladder." Journal of Anatomy **136**(Pt 2): 265-271.

Dixon, J.S. & Jen P.Y. (1995). "Development of nerves containing nitric oxide synthase in the human male urogenital organs." British Journal of Urology **76**(6): 719-25.

Dmochowski, R.R., Miklos J.R., et al. (2003). "Duloxetine versus placebo for the treatment of North American women with stress urinary incontinence." Journal of Urology **170**(4 Pt 1): 1259-1263.

Downie, J.W. & Karmazyn M. (1984). "Mechanical trauma to bladder epithelium liberates prostanoids which modulate neurotransmission in rabbit detrusor muscle." Journal of Pharmacology and Experimental Therapeutics **230**(2): 445-449.

Doran, C.M., Chiarelli P. & Cockburn J. (2001). "Economic costs of urinary incontinence in community-dwelling Australian women." The Medical Journal of Australia **174**(9): 456-458.

Drumm, B.T., Large R.J., et al. (2015). "The role of Ca(2+) influx in spontaneous Ca(2+) wave propagation in interstitial cells of Cajal from the rabbit urethra." Journal of Physiology **593**(15): 3333-3350.

Dubeau, C.E., Kraus S.R., et al. (2014). "Effect of fesoterodine in vulnerable elderly subjects with urgency incontinence: a double-blind, placebo controlled trial." Journal of Urology **191**(2): 395-404.

Duffy, A.M., Cobine C.A. & Keef K.D. (2012). "Changes in neuromuscular transmission in the W/W(v) mouse internal anal sphincter." Neurogastroenterology and Motility **24**(1): e41-e55.

Dupuis, M., Houdeau E. & Mhaouty-Kodja S. (2008). "Increased potency of alpha 1-adrenergic receptors to induce inositol phosphates production correlates with the up-

regulation of alpha 1d/Gh alpha/phospholipase C delta 1 signaling pathway in term rat myometrium." Reproduction **135**(1): 55-62.

Durlu-Kandilci, N. T., & Brading, A. F. (2006). "Involvement of Rho kinase and protein kinase C in carbachol-induced calcium sensitization in beta-escin skinned rat and guinea-pig bladders." British Journal of Pharmacology **148**(3): 376-384.

Dutton, J.L., Hansen M.A., et al. (1999). "Development of P2X receptor clusters on smooth muscle cells in relation to nerve varicosities in the rat urinary bladder." Journal Neurocytology **28**(1): 4-16.

Duringer, C., Grundstrom G., et al. (2009). "Agonist-specific patterns of beta 2-adrenoceptor responses in human airway cells during prolonged exposure." British Journal of Pharmacology **158**(1): 169-179.

Ecke, D., Hanck T., et al. (2008). "Hetero-oligomerization of the P2Y11 receptor with the P2Y1 receptor controls the internalization and ligand selectivity of the P2Y11 receptor." The Biochemical Journal **409**(1): 107-116.

Ehlert, F.J., Ahn S., et al. (2007). "Neuronally released acetylcholine acts on the M2 muscarinic receptor to oppose the relaxant effect of isoproterenol on cholinergic contractions in mouse urinary bladder." Journal of Pharmacology and Experimental Therapeutics **322**: 631–637.

Ehren, I., Iversen H., et al. (1994). "Localization of nitric oxide synthase activity in the human lower urinary tract and its correlation with neuroeffector responses." Urology **44**(5):683-687.

Ek, A., Andersson K.E., et al. (1978). "The effects of long-term treatment with norephedrine on stress incontinence and urethral closure pressure profile." Scandinavian Journal of Urology and Nephrology **12**(2): 105-110.

Ek, A., Alm, P., et al. (1977). "Adrenoceptor and cholinoceptor mediated responses of the isolated human urethra." Scandinavian Journal of Urology and Nephrology **11**(2): 97-102.

Emorine, L.J., Marullo S., et al. (1989). "Molecular characterization of the human beta 3-adrenergic receptor." Science **245**(4922): 1118-1121.

Engel, A.F., Kamm M.A., et al. (1995). Relationship of symptoms in faecal incontinence to specific sphincter abnormalities. International Journal of Colorectal Disease **10**(3): 152–155.

Engelhardt, S., Zieger W., et al. (1997). "Tocolytic therapy with fenoterol induces selective downregulation of ß -adrenergic receptors in human myometrium." Journal of Clinical Endocrinology Metabolism **82**: 1235–1242.

Ercan, O., Kostu B., et al. (2015). "Comparison of solifenacin and fesoterodine in treatment of overactive bladder." Saudi Medical Journal **36**(10): 1181-1185.

Escriche, M., Burgueno J., et al. (2003). “Ligand-induced caveolae-mediated internalization of A1 adenosine receptors: morphological evidence of endosomal sorting and receptor recycling.” Experimental Cell Research **285**(1): 72-90.

Evers, J., Buffini M. et al. (2013). “Is there a nitrergic modulation of the rat external anal sphincter?” Experimantal Physiology **98**(2): 397-404.

Fan, Y.P., Chakder S., et al. (2001). “Inducible and neuronal nitric oxide synthase involvement in lipopolysaccharide-induced sphincteric dysfunction.” American Journal of Physiology; Gastrointestinal and liver physiology **280**(1): G32-G42.

Farid, M., El Nakeeb A., et al. (2009). “Idiopathic hypertensive anal canal: a place of internal sphincterotomy.” Journal of Gastrointestinal surgery **13**(9): 1607-1613.

Farouk, R., Duthie G.S., et al. (1994). “Sustained internal sphincter hypertonia in patients with chronic anal fissure.” Diseases of the Colon and Anorectum **37**(5): 424-429.

Ferguson, S. S. (2001). “Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling.” Pharmacological Review **53**(1): 1-24.

Ferguson, D.R., Kennedy I. & Burton T.J. (1997). “ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes--a possible sensory mechanism?” Journal of Physiology **505** (Pt 2): 503-511.

Ferguson, A.C., Sutton B.W., et al. (2015). “Inhibition of urothelial P2X3 receptors prevents desensitization of purinergic detrusor contractions in the rat bladder.” British Journal of Urology International **116**(2): 293-301.

Fernandes, V.S., Ribeiro A.S., et al. (2013). “Hydrogen sulfide mediated inhibitory neurotransmission to the pig bladder neck: role of KATP channels, sensory nerves and calcium signaling.” Journal of Urology **190**(2): 746-756.

Fernández-Fraga, X., Azpiroz F. & Malagelada J.R. (2002). “Significance of pelvic floor muscles in anal incontinence.” Gastroenterology **123**(5): 1441-1450.

Farrugia, G., Irons W.A., et al. (1993). Activation of whole cell currents in isolated human jejunal circular smooth muscle cells by carbon monoxide. American Journal of Physiology **264**(6pt1): G1184–G1189.

Fetscher, C., Fleichman M., et al. (2002). “M(3) muscarinic receptors mediate contraction of human urinary bladder.” British Journal of Pharmacology **136**(5): 641-643.

Fiehn, O. & Kim J. (2014). “Metabolomics insights into pathophysiological mechanisms of interstitial cystitis.” International Neurourology Journal **18**(3): 106-114.

Fink, K.G., Huber J., et al. (2008). “The use of Duloxetine in the treatment of male stress urinary incontinence.” Wiener Medizinische Wochenschrift **158**(3-4): 116-118.

Finney, P.A., Donnelly L.E., et al. (2001). "Chronic systemic administration of salmeterol to rats promotes pulmonary beta(2)-adrenoceptor desensitization and down-regulation of G(s alpha)." British Journal of pharmacology **132**(6): 1261-1270.

Fleichman, M., Schneider, T., et al. (2004). "Signal transduction underlying carbachol-induced contraction of rat urinary bladder. II. Protein kinases." Journal of Pharmacology and Experimental Therapeutics **308**(1): 54-58.

Fogli, S., Pellegrini S., et al. (2011). "Rosiglitazone reverses salbutamol-induced ß(2) -adrenoceptor tolerance in airway smooth muscle." Britsih Journal of Pharmacology **162**(2): 378-391.

Folasire, O., Mills K.A. et al. (2016). "Three Gaseous Neurotransmitters, Nitric oxide, Carbon Monoxide, and Hydrogen Sulfide, Are Involved in the Neurogenic Relaxation Responses of the Porcine Internal Anal Sphincter." Journal of Neurogastroenterology **22**(1):141-148.

Ford, A.P., Williams T.J., et al. (1994). "Alpha 1-adrenoceptor classification: sharpening Occam's razor." Trends in Pharmacological Sciences **15**: 167–170.

Ford A.P., Arredondo N.F., et al. (1996). RS-17053 (N-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro-alpha, alpha-dimethyl-1H-indole-3-ethanamine hydrochloride), a selective alpha 1A-adrenoceptor antagonist, displays low-affinity for functional alpha 1-adrenoceptors in human prostate: implications for adrenoceptor classification. Molecular pharmacology **49**(2): 209–215.

Fovaeus, M., Fujiwara, M., et al. (1998). "A non-nitrergic smooth muscle relaxant factor released from the contracting rat urinary bladder." Acta physiologica and scandinavica **162**(1): 116-116.

Fovaeus, M., Fujiwara, M., et al. (1999). "A non-nitrergic smooth muscle relaxant factor released from rat urinary bladder by muscarinic receptor stimulation." Journal of Urology **161**(2): 649-653.

Fowler, C. J., Griffiths, D., & de Groat, W. C. (2008). "The neural control of micturition. Nature reviews." Neuroscience **9**(6): 453-466.

Francis, S. H., Busch, J. L., et al. (2010). "cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action." Pharmacological Reviews **62**(3): 525-563.

Frazier, E.P., Braverman A.S., et al. (2007). "Does phospholipase C mediate muscarinic receptor-induced rat urinary bladder contraction?" Journal of Pharmacology and Experimental Therapeutics **322**(3): 998–1002.

Frazier, E.P., Mathy M.J., et al. (2005). "Does cyclic AMP mediate rat urinary bladder relaxation by isoproterenol?" Journal of Pharmacology and Experimental Therapeutics **313** (1): 260–267.

Frazier, E.P., Peters S.L.M., et al. (2008). "Signal transduction underlying control of urinary bladder smooth muscle tone by muscarinic receptors and b-adrenoceptors." Naunyn-Schmiedeberg's Archives of Pharmacology **377** (4-6): 449–462.

Fredholm, B.B., Abbracchio M.P., et al. (1994). "Nomenclature and classification of purinoceptors." Pharmacological Reviews **46**(2): 143-156.

Frenckner, B. & Euler C.V. (1975). Influence of pudendal block on the function of the anal sphincters. Gut **16**(6): 482–489.

Fry, C.H. & Young J. S. (2010). "The physiology and pharmacology of the lower urinary tract." Surgery **28**(7): 317-322.

Fu L.X., Liang Q.M., et al. (1992). "Increase in functional activity rather than in amount of Gi-alpha in failing human heart with dilated cardiomyopathy." Cardiovascular Research **26**(10): 950-955.

Fukata, A. & Yamazaki T. (2016). "Comparative Influence of Imidafenacin and Oxybutynin on Voiding Function in Rats with Functional Urethral Obstruction." Drug Research **66**(6): 306-11.

Furchgott, R.F. & Zawadzki J.V. (1980). "The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine." Nature **288**(5789): 373-376.

Furness, J.B. (2015). "Enteric Nervous System: Structure, Relationships and Functions." Reference Module in Biomedical Sciences. doi:10.1016/B978-0-12-801238-3.04584-0.

Fynne, L., Worsoe J. et al. (2011). Faecal incontinence in patients with systemic sclerosis: is an impaired internal anal sphincter the only cause? Scandinavian Journal of Rheumatology **40**(6): 462-466.

Gabella, G. & Davis C. (1998). "Distribution of afferent axons in the bladder of rats." Journal of Neurocytology **27**(3): 141-155.

Gai, J. W., Wahafu, W., et al. (2013). "Further evidence of endogenous hydrogen sulphide as a mediator of relaxation in human and rat bladder." Asian Journal of Andrology **15**(5): 692-696.

Gandomkar, H., Zeinoddini A., et al. (2015). "Partial lateral internal sphincterotomy versus combined botulinum toxin A injection and topical diltiazem in the treatment of chronic anal fissure: a randomized clinical trial." Diseases of the Colon and Rectum **58**(2): 228-234.

Gao, H., Sun Y., et al. (2004). "Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-kappaB pathways." Molecular Cell **14**(3): 303-317.

Gao, N., Chang A.N., et al. (2016). "Physiological signalling to myosin phosphatase targeting subunit-1 phosphorylation in ileal smooth muscle." Journal of Physiology **594**(12): 3209-25.

Garcia-Pascual, A., Costa G., et al. (1996). "Characterization of nitric oxide synthase activity in sheep urinary tract: functional implications." British Journal of Pharmacology **118**(4): 905-914.

Garcia-Sacristan, A., Casanueva C. R., et al. (1984). "Adrenergic receptors in the urethra and prostate of the horse." Research in Veterinary Science **36**(1): 57-60.

Garcia-Sainz, J. A., Vazquez-Prado J., & del Carmen Medina L. (2000). "Alpha 1-adrenoceptors: function and phosphorylation." European Journal of Pharmacology **389**(1): 1-12.

Garg, P. (2010). "Water stream in a bidet-toilet as a cause of anterior fissure-in-ano: a preliminary report." Colorectal Diseases **12**(6): 601-602.

Ge, J., Yang P., et al. (2015). "Prevalence and risk factors of urinary incontinence in Chinese women: a population-based study." Asian pacific Journal of Public Health **27**(2): NP1118-1131.

Gerthoffer, W.T., Murphey K.A., et al. (1991). "Myosin phosphorylation and calcium in tonic and phasic contractions of colonic smooth muscle." American Journal of Physiology **260**(6 pt 1):G958-G964.

Gevaert, T., De Vos R., et al. (2011). "Characterization of upper lamina propria interstitial cells in bladders from patients with neurogenic detrusor overactivity and bladder pain syndrome." Journal of Cellular and Molecular Medicine **15**(12): 2586-2593.

Gevaert, T., De Vos R., et al. (2012). "Identification of telocytes in the upper lamina propria of the human urinary tract." Journal of Cellular and Molecular Medicine **16**(9): 2085-2093.

Gibbons, C.P., Bannister J.J., et al. (1986a). An analysis of anal sphincter pressure and anal compliance in normal subjects. International Journal of Colorectal Disease **1**(4) 231–237.

Gibbons, C.P., Trowbridge., et al. (1986b). Role of anal cushions in maintaining continence. Lancet **1**(8486): 886–888.

Giglio, D. & Tobin G. (2009). "Muscarinic receptor subtypes in the lower urinary tract." Pharmacology **83**(5): 256-269.

Gil, V., Gallego D. & Jiménez M. (2011). "Effects of inhibitors of hydrogen sulphide synthesis on rat colonic motility." British Journal of Pharmacology **164**(2b): 485-498.

Gillespie, J.I., Markerink-van Ittersum M., et al. (2005). "Expression of neuronal nitric oxide synthase (nNOS) and nitric-oxide-induced changes in cGMP in the urothelial layer of the guinea pig bladder." Cell and Tissue Research **321**(3): 341-351.

Gimenez, L.E., Baameur F., et al. (2015). "Salmeterol Efficacy and Bias in the Activation and Kinase-Mediated Desensitization of ß2-Adrenergic Receptors." Molecular Pharmacology **87**(6): 954-964.

Ginsberg, D., Schneider T., et al. (2013). “Efficacy of fesoterodine compared with extended-release tolterodine in men and women with overactive bladder.” British Journal of Urology International **112**(3): 373-385.

Glavind, E.B., Forman A., et al. (1997). “Effects of transmural field stimulation in isolated smooth muscle of human rectum and internal anal sphincter.” American Journal of Physiology **272**(5 Pt 1): G1075-G1082.

Goepel, M., Wittmann A., et al. (1997). “Comparison of adrenoceptor subtype expression in porcine and human bladder and prostate.” Urological Research **25**(3): 199-206

Goldstein, S. R., Johnson S., et al. (2005). “Incidence of urinary incontinence in postmenopausal women treated with raloxifene or estrogen.” Menopause **12**(2): 160-164.

Gonzalez, E.J., Merrill L., et al. (2014). “Bladder sensory physiology: neuroactive compounds and receptors, sensory transducers, and target-derived growth factors as targets to improve function.” American Journal of Physiology; Regulatory, Integrative and Comaparative Physiology **306**(12): R869-R878.

González-Arenas, A., Aguilar-Maldonado B., et al. (2006). “Estrogens cross-talk to alpha1b-adrenergic receptors.” Molecular Pharmacology **70**(1): 154-162.

GonzÁLez-Soriano, J., MartÍN-Palacios S., et al. (2003). “Nitric Oxide Synthase in the External Urethral Sphincter of the Sheep: Immunohistochemical and Functional Study.” The Journal of Urology **169**(5): 1901-1906.

Goral, V., Jin Y., et al. (2011). "Agonist-directed desensitization of the β2-adrenergic receptor." Plos One **6**(4):e19282.

Gordon P.H. & Nivatvongs S. (2007). “Principles and practice of surgery for the colon, rectum, and anus.” Informa Healthcare 3rd edition page **8**.

Gosling, J.A. & Dixon J.S. (1974). “Sensory nerves in the mammalian urinary tract. An evaluation using light and electron microscopy.” Journal of Anatomy **117** (pt 1): 133-144.

Gosling, J.A (1997). “Modification of bladder structure in response to outflow obstruction and ageing.” European Urology 32 (Suppl 1): 9-14.

Goyal, R.K., Rattan S. & Said S.I. (1980). "VIP as a possible neurotransmitter of non-cholinergic non-adrenergic inhibitory neurones." Nature **288**(5789):378-380.

Graham, R. M., Perez, D. M., et al. (1996). “Alpha 1-adrenergic receptor subtypes. Molecular structure, function, and signaling.” Circulation Research **78**(5): 737-749.

Grandordy, B. M., Mak, J. C., & Barnes, P. J. (1994). “Modulation of airway smooth muscle beta-adrenoceptor function by a muscarinic agonist.” Life Science **54**(3): 185-191.

Gray, K., Short J. & Ventura S. (2008). "The alpha1A-adrenoceptor gene is required for the alpha1L-adrenoceptor-mediated response in isolated preparations of the mouse prostate." British Journal of Pharmacology **155**(1): 103-109.

Gray, S.L., Anderson M.L., et al. (2015). "Cumulative use of strong anticholinergics and incident dementia: a prospective cohort study." JAMA Internal Medicine 175(3): 401-407.

Greenland, J. E., Dass N., & Brading A. F. (1996). "Intrinsic urethral closure mechanisms in the female pig." Scandinavian Journal of Urology and Nephrology **179**: 75-80.

Grol, S., van Koeveringe G.A., et al. (2008). "Regional differences in sensory innervation and suburothelial interstitial cells in the bladder neck and urethra." British Journal of Urology International **102**: 870–877.

Gruss, H.J., Pediconi C. & Jacobs A. (2014). "Meta-analysis for cardiovascular effects of NRL001 after rectal application in healthy volunteers.Colorectal Diseases **16** (Suppl 1): 51-58.

Guan, N.N., Thor A., et al. (2014a). "Cascade bioassay evidence for the existence of urothelium-derived inhibitory factor in Guinea pig urinary bladder." Plos One **9**(8): e103932.

Guan, N.N., Nilsson K.F., et al. (2014b). "Release and inhibitory effects of prostaglandin D2 in guinea pig urinary bladder and the role of urothelium." Biochimica et biophysica Acta **1840**(12): 3443-3451.

Gur, S., Kadowitz P. J., & Hellstrom W. J. (2011). "RhoA/Rho-kinase as a therapeutic target for the male urogenital tract." Journal of Sexual Medicine **8**(3): 675-687.

Gustafson, K.J., Creasey G.H. & Grill W.M. (2003). "A catheter based method to activate urethral sensory nerve fibers." Urology **170**(1): 126-129.

Gutierrez, J.G. & Shah A.N. (1975). Autonomic control of the internal anal sphincter in man. In: Trappen G, editor. Vth International Symposium on Gastrointestinal Motility. Leuven: Typoff Press; pp. 363–373.

Haas, M., Shaaban A. & Reiser G. (2014). "Alanine-(87)-threonine polymorphism impairs signaling and internalization of the human P2Y11 receptor, when co-expressed with the P2Y1 receptor." Journal of Neurochemistry **129**(4): 602-613.

Habler, H.J., Janig W. & Koltzenburg M. (1990). "Activation of unmyelinated afferent fibres by mechanical stimuli and inflammation of the urinary bladder in the cat." Journal of Physiology **425**: 545-562.

Habler, H.J., Jänig W. & Koltzenburg M. (1993). "Receptive properties of myelinated primary afferents innervating the inflamed urinary bladder of the cat." Journal of Neurophysiology **69**(2): 395-405.

Hague, C., Uberti M. A., et al. (2004a). “Cell surface expression of a1D-adrenergic receptors is controlled by heterodimerization with a1B-adrenergic receptors. Journal of Biological Chemistry **279** (15): 15541–15549.

Hague, C., Uberti M.A., et al. (2004b). “Olfactory receptor surface expression is driven by association with the beta2-adrenergic receptor.” Proceeding of the National Academy of Sciences of the United States of America **101**(37): 13672-13676.

Hakenberg, O.W., Linne C., et al. (2000). “Bladder wall thickness in normal adults and men with mild lower urinary tract symptoms and benign prostatic enlargement.” Neurourology and Urodynamics **19**(5): 585–593.

Han, E., Black L.K. & Lavelle J.P. (2007). “Incontinence related to management of benign prostatic hypertrophy.” The American Journal of Geriatric Pharmacotherapy **5**(4): 324-334.

Hananel, N. & Gordon P.H. (1997). Re-examination of clinical manifestations and response to therapy of fissure-in-ano. Diseases of the Colon and Rectum **40**(2): 229-233.

Hanna-Mitchell, A.T., Beckel J.M., et al. (2007). “Non-neuronal acetylcholine and urinary bladder urothelium.” Life Sciences **80**(24-25): 2298-2302.

Harraz, O.F. & Altier C. (2014). “STIM1-mediated bidirectional regulation of Ca(2+) entry through voltage-gated calcium channels (VGCC) and calcium-release activated channels (CRAC).” Frontiers in cellular Neurosciences **24**: 8-23.

Harriss, D.R., Marsh K.A., et al. (1995). “Expression of muscarinic M3-receptors coupled to inositol phospholipid hydrolysis in human detrusor cultured smooth muscle cells.” Journal of Urology **154**: 1241–1245.

Harrison, M.R., Ross N., et al., (1983). "Correction of congenital hydronephrosis in utero. I. The model: fetal urethral obstruction produces hydronephrosis and pulmonary hypoplasia in fetal lambs." Journal of Pediatric Surgery **18**(3):247-256.

Hashimoto, Y., Ushiki T., et al. (1999). “Scanning electron microscopic observation of apical sites of open-type paraneurons in the stomach, intestine and urethra.” Archives of Histology and Cytology **62**(2): 181–189.

Hashitani, H. & Suzuki H. (2007). “Properties of spontaneous Ca2+ transients recorded from interstitial cells of Cajal-like cells of the rabbit urethra in situ.” The Journal of Physiology **583**(pt2): 505-519.

Hashitani, H., Yanai Y. & Suzuki H. (2004a). “Role of interstitial cells and gap junctions in the transmission of spontaneous Ca2+ signals in detrusor smooth muscles of the guinea-pig urinary bladder.” Journal of Physiology **559**(pt2): 567-581.

Hashitani, H., Brading A.F. & Suzuki H. (2004b). “Correlation between spontaneous electrical, calcium and mechanical activity in detrusor smooth muscle of the guinea-pig bladder.” British Journal of Pharmacology **141**(1): 183-193.

Hashitani, H., Fukuta H., et al. (2001). “Origin and propagation of spontaneous excitation in smooth muscle of the guinea-pig urinary bladder.” Journal of Physiology **530**(Pt 2): 273-286.

Hashitani, H., Yanai Y., et al. (2006). “Heterogeneous CPA sensitivity of spontaneous excitation in smooth muscle of the rabbit urethra.” British Journal of Pharmacology **148**(3): 340-349.

Hashitani, H., Takano H., et al. (2011). “Functional properties of suburothelial microvessels in the rat bladder.” Journal of Urology **185**(6): 2382-2391.

Hawthorn, M. H., Chapple, C. R., et al. (2000). “Urothelium-derived inhibitory factor(s) influences on detrusor muscle contractility in vitro.” British Journal of Pharmacology **129**(3): 416-419.

Hayashi, N., Sugimura Y., et al. (1991). Morphological and functional heterogeneity in the rat prostatic gland. Biology of Reproduction **45**(2): 308-321.

Hayashi, M., Kajioka S., et al. (2016). “Actions of cAMP on calcium sensitization in human detrusor smooth muscle contraction.” British Journal of urology **117**(1): 179-191.

Haylen, B. T., de Ridder, D., et al. (2010). “An international urogynecological association (IUGA)/international continence society (ICS) joint report on the terminology for female pelvic floor dysfunction.” Neurourology and Urodynamics **29**(1): 4-20.

He, W.Q., Peng Y.J, et al. (2008a). “Myosin light chain kinase is central to smooth muscle contraction and required for gastrointestinal motility in mice.” Gastroenterology **135**(2): 610-620.

He, Y., Xu, H., et al. (2008b). “Antiinflammatory effect of Rho kinase blockade via inhibition of NF-kappaB activation in rheumatoid arthritis.” Arthritis and Rheumatology **58**(11): 3366-3376.

Heckler, M.M., Thakor H., et al. (2014). “ERK/MAPK regulates ERRϒ Expression, transcriptional activity and receptor-mediated tamoxifen resistance in ER+ breast cancer.” FEBS letter **28**1(10): 2431-2442.

Hegde, S.S. (2006). “Muscarinic receptors in the bladder: from basic research to therapeutics.” British Journal of Pharmacology **147**:S80–S87.

Hegde, S.S., Mandel D.A., et al. (1993). "Evidence for purinergic neurotransmission in the urinary bladder of pithed rats." European Journal of Pharmacology **349**(1):75-82.

Hegde, S.S., Choppin A., et al. (1997). “Functional role of M2 and M3 muscarinic receptors in the urinary bladder of rats in vitro and in vivo.” British Journal of Pharmacology **120**(8): 1409-1418.

Heinrich, M.C., Corless C.L. et al. (2003). “Immunohistochemical study of c-KIT (CD117) expression in renal cell carcinoma.” Journal of Clinical Oncology **21**(3): 4342-4349.

Hennenberg, M., Strittmatter F., et al. (2011). “a1-adrenoceptor activation induces phosphorylation of ß2-adrenoceptors in human prostate tissue.” British Journal of Urology International **108**(6): 922-928.

Hennig, B. & Diener M. (2009). “Actions of hydrogen sulphide on ion transport across rat distal colon.” British Journal of Pharmacology **158**(5): 1263-1275.

Hernandez, M., Knight G.E., et al. (2009). “Role of ATP and related purines in inhibitory neurotransmission to the pig urinary bladder neck.” British Journal of Pharmacology **157**(8): 1463-1473.

Herschorn, S., Barkin J., et al. (2013). “A phase III, randomized, double-blind, parallel-group, placebo-controlled, multicentre study to assess the efficacy and safety of the ß3 adrenoceptor agonist, mirabegron, in patients with symptoms of overactive bladder.” Urology **82**(2): 313-320.

Herzig, D.O. & Lu K.C. (2010). “Anal fissure.” Surgical Clinics of North America **90**(1): 33-44.

Hey, C., Wessler I., & Racké K. (1994). “Muscarinic inhibition of endogenous noradrenaline release from rabbit isolated trachea: receptor subtype and receptor reserve.” Naunyn-Schmiedeberg's archives of Pharamcology **350**(5): 464-472.

Hien, T.T., Turczynska K.M., et al. (2016). “Elevated Glucose Levels Promote Contractile and Cytoskeletal Gene Expression in Vascular Smooth Muscle via Rho/Protein Kinase C and Actin Polymerization.” Journal of Biological Chemistry **291**(7): 3552-3568.

Hill, W. G. (2015). “Control of urinary drainage and voiding.” Clinical Journal of American Society of Nephrology **10**(3): 480-492.

Himpens, B., Missiaen L., et al. (1991). “AlF4- induces Ca2+ oscillations in guinea-pig ileal smooth muscle.” Pfluger's Archives **417**(6): 645-650.

Hirata, M., Kohse K.P., et al. (1990). “Mechanism of cyclic GMP inhibition of inositol phosphate formation in rat aorta segments and cultured bovine aortic smooth muscle cells.” The Journal of Biological Chemistry **265**(3): 1268-1273.

Hiraoka, Y., Ohmura T., et al. (1995). “Identification of alpha 1-adrenoceptor subtypes in the rabbit prostate.” Journal of Autonomic Pharmacology **15**(4): 271-278.

Ho, K. M., Borja, M. C., et al. (2003). “Expression of nitric oxide synthase immunoreactivity in the human female intramural striated urethral sphincter.” Journal of Urology **169**(6): 2407-2411.

Ho, K.M., McMurray G., et al. (1998). "Nitric oxide synthase in the heterogeneous population of intramural striated muscle fibres of the human membranous urethral sphincter." Journal of Urology **159**(3):1091-1096.

Hofmann, F. (2005). "The biology of cyclic GMP-dependent protein kinases." Journal of Biological chemistry **280**(1):1-4.

Hokfelt, T., Schultzberg M. et al., (1978). "Peptide neurons in peripheral tissues including the urinary tract: immunohistochemical studies." Acta Pharmacologica et Toxicologia **43**(2): 79-89.

Hollywood, M.A., Woolsey S., et al. (2003a). "T- and L-type Ca2+ currents in freshly dispersed smooth muscle cells from the human proximal urethra." The Journal of Physiology **550**(pt 3): 753-764.

Hollywood, M.A., Sergeant G.P., et al. (2003b). "Activation of Ca2+-activated Cl- current by depolarizing steps in rabbit urethral interstitial cells." American Journal of Physiology; Cell Physiology **285**(2): C327-C333.

Hosey, M.M., Benovic J.L., et al. (1995). "Multiple mechanisms involving protein phosphorylation are linked to desensitization of muscarinic receptors." Life Sciences **56**(11-12): 951-955.

Hosoki, R., Matsuki N. & Kimura H. (1997). "The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide." Biochemical and Biophysical Research Communication **237**(3): 527-531.

Hristov, K.L., Cui X., et al. (2008). "Stimulation of beta3-adrenoceptors relaxes rat urinary bladder smooth muscle via activation of the large-conductance Ca2+-activated K+ channels." American Journal of Physiology; Cell Physiology **295**(5): C1344-C1353.

Hsieh, P.F., Chiu H.C., et al. (2016). "Botulinum toxin A for the Treatment of Overactive Bladder." Toxins **8**: 3.

Hudson, C.N., Sohaib S.A., et al. (2002). "The anatomy of the perineal membrane: its relationship to injury in childbirth and episiotomy." The Australian and New Zealand Journal of Obstetrics & gynaecology **42**(2): 193–196.

Hudson, B.D., Hébert T.E. & Kelly M.E. (2010). "Physical and functional interaction between CB1 cannabinoid receptors and beta2-adrenoceptors." British Journal of Pharmacology **160**(3): 627-642.

Hunskaar, S., Lose G., et al., (2004). "The prevalence of urinary incontinence in women in four European countries." British Journal of Urology International **93** (3): 324-330.

Hypolite, J. A., Chang S., et al. (2015). "Protein kinase C modulates frequency of micturition and non-voiding contractions in the urinary bladder via neuronal and myogenic mechanisms." Biomedical Central Urology **15**: 34.

Iaccarino, G., Barbato E., et al. (2005). "Elevated myocardial and lymphocyte GRK2 expression and activity in human heart failure." European Heart Journal **26**(17): 1752-1758.

Igawa, Y., Mattiasson A. & Andersson K.E. (1993). "Functional importance of cholinergic and purinergic neurotransmission for micturition contraction in the normal, unanaesthetized rat." British Journal of Pharmacology **109**(2):473-479.

Igawa, Y., Yamazaki Y., et al. (1999). "Functional and molecular biological evidence for a possible beta3-adrenoceptor in the human detrusor muscle." British Journal of Pharmacology **126**(3): 819-825.

Igawa, Y., Yamazaki Y., et al. (2001). "Relaxant effects of isoproterenol and selective beta3-adrenoceptor agonists on normal, low compliant and hyperreflexic human bladders." Journal of Urology **165**(1): 240-244.

Igawa, Y., Kumano S., et al. (2014). "Changes in the function and expression of T-type and N-type calcium channels in the rat bladder after bladder outlet obstruction." Journal of Urology **191**(4): 1159-1167.

Iijima, K., De Wachter S. & Wyndaele J.J. (2007). "Effects of the M3 receptor selective muscarinic antagonist darifenacin on bladder afferent activity of the rat pelvic nerve." European Urology **52**: 842–847.

Ikenouchi, T., Kume H., et al. (2008). "Role of Ca(2+) mobilization in desensitization of beta-adrenoceptors by platelet-derived growth factor in airway smooth muscle." European Journal of Pharmacology **591**(1-3): 259-265.

Inoue, R. & Isenberg G. (1990). "Acetylcholine activates nonselective cation channels in guinea pig ileum through a G protein. American Journal of Physiology **258**(6 Pt 1): C1173-C1178.

Irwin, D.E., Kopp Z.S., et al. (2011). "Worldwide prevalence estimates of lower urinary tract symptoms, overactive bladder, urinary incontinence and bladder outlet obstruction." British Journal of Urology International **108**(7): 1132-1138.

Ishihama, H., Momota Y., et al. (2006) "Activation of alpha1D adrenergic receptors in the rat urothelium facilitates the micturition reflex." Journal of Urology **175**(1): 358-364.

Ishiko, O., Hirai K. et al. (2001). Hormone replacement therapy plus pelvic floor muscle exercise for postmenopausal stress incontinence. A randomized, controlled trial. Journal of Reproductive Medicine **46**(3): 213-220.

Ishiyama G., Hinata N., et al. (2014). "Nerves supplying the internal anal sphincter: an immunohistochemical study using donated elderly cadavers." Surgical and Radiologic Anatomy **36**(10):1033-1042.

Isotani, E., Zhi G., et al. (2004). "Real-time evaluation of myosin light chain kinase activation in smooth muscle tissues from a transgenic calmodulin-biosensor mouse." Proceedings of National Academy of Science USA **101**(16): 6279-6284.

Ito, Y., & Kimoto Y. (1985). "The neural and non-neural mechanisms involved in urethral activity in rabbits. Journal of Physiology **367**: 57-72.

Ito, K., Shimomura E., et al. (2003). "Essential role of rho kinase in the Ca2+ sensitization of prostaglandin F(2alpha)-induced contraction of rabbit aortae." Journal of Physiology **546**(Pt 3): 823-836.

Janig, W. & Morrison J.F. (1986). "Functional properties of spinal visceral afferents supplying abdominal and pelvic organs, with special emphasis on visceral nociception." Progress in Brain Research **67**: 87-114.

January, B., Seibold A., et al. (1997). "beta2-adrenergic receptor desensitization, internalization, and phosphorylation in response to full and partial agonists." Journal of Biological Chemistry **272**(38): 23871-23879.

January, B., Seibold A., et al. (1998). "Salmeterol-induced desensitization, internalization and phosphorylation of the human beta2-adrenoceptor." British Journal of Pharmacology **123**(4): 701-11.

Jayarajan J. & Radomski S.B. (2013). "Pharmacotherapy of overactive bladder in adults: a review of efficacy, tolerability, and quality of life." Research and Reports in Urology **6**:1-6.

Jeong, M.S. & Lee J.G. (2000). "The role of spinal and peripheral alpha1- and alpha2-adrenoceptors on bladder activity induced by bladder distension in anaesthetized rat." British Journal of Urology International **85**(7): 925-931.

Jeremy, J. Y., Tsang V., et al. (1987). "Eicosanoid synthesis by human urinary bladder mucosa: Pathological implications." British Journal of Urology **59**(1): 36-39.

Jiang, M.T., Moffat M.P & Narayanan N. (1993). "Age-related alterations in the phosphorylation of sarcoplasmic reticulum and myofibrillar proteins and diminished contractile response to isoproterenol in intact rat ventricle. Circulation Research **72**(1): 102-11.

Jiang, X., Luttrell I., et al. (2014). "T- and L-type voltage-gated calcium channels: their role in diabetic bladder dysfunction." Neurourology and urodynamics **33**(1): 147-152.

Jiang, X., Pan H., et al. (2012). "A novel EST-derived RNAi screen reveals a critical role for farnesyl diphosphate synthase in ß2-adrenergic receptor internalization and down-regulation." FASEB Journal **26**(5): 1995-2007.

Johnson, E. A., Oldfield S., et al. (2006). "Agonist-selective mechanisms of mu-opioid receptor desensitization in human embryonic kidney 293 cells." Molecular Pharmacology **70**(2): 676-685.

Johnston, L., Carson C., et al. (2008). "Cholinergic-induced Ca2+ signaling in interstitial cells of Cajal from the guinea pig bladder." American Journal of Renal Physiology **294**(3): F645-F655.

Johnston, L., Woolsey S. et al. (2010). “Morphological expression of KIT positive interstitial cells of Cajal in human bladder.” Journal of Urology **184** (1): 370-377.

Jonas, M., Neal K.R. et al. (2001). A randomized trial of oral vs. topical diltiazem for chronic anal fissures. Diseases of the Colon and Rectum **44**(8):1074-1078.

Jones, O.M., Brading A.F. & Mortensen N.J. (2003a). “Role of nitric oxide in anorectal function of normal and neuronal nitric oxide synthase knockout mice: a novel approach to anorectal disease.” Diseases of the Colon and Rectum **46**(7): 963-970.

Jones, O.M., Thompson J.M., et al. (2003b). "L-Erythro-methoxamine is more potent than phenylephrine in effecting contraction of internal anal sphincter in vitro." British Journal of Surgery **90**(7):872-876.

Jost, S.P., Gosling J.A. & Dixon J.S. (1989). “The morphology of normal human bladder urothelium.” Journal of Anatomy **167**: 103-115.

Jost, W.H. (1997). One hundred cases of anal fissure treated with botulin toxin: early and long-term results. Diseases of the Colon and Rectum **40**(9): 1029–1032.

Julia-Guilloteau, V., Denys P., et al. (2007). “Urethral closure mechanisms during sneezing-induced stress in anesthetized female cats.” American Journal of Physiology; Regulatory, Integrative and Comparative Physiology **293**(3): R1357-R1367.

Jung, S.Y., Fraser M.O., et al. (1999). “Urethral afferent nerve activity affects the micturition reflex; implication for the relationship between stress incontinence and detrusor instability.” Journal of Urology **162**(1): 204-212.

Kabil, O., Vitvitsky V., et al. (2011). “The quantitative significance of the transsulfuration enzymes for H2S production in murine tissues.” Antioxidants and Redox Signaling **15**(2): 363-372.

Kaiho, Y., Kamo I., et al. (2007). “Role of noradrenergic pathways in sneeze-induced urethral continence reflex in rats.” American Journal of Physiology; Renal Physiology **292**(2): F639-F346.

Kajioka, S., Nakayama S., et al. (2002). “Ca(2+) channel properties in smooth muscle cells of the urinary bladder from pig and human.” European Journal of Pharmacology **443**(1-3): 19-29.

Kakizaki, H., Fraser M.O., et al. (1997). “Reflex pathways controlling urethral striated and smooth muscle function in the male rat.” American Journal of Physiology **272**: R1647–R1656.

Kamo, I., Torimoto K., et al. (2003). “Urethral closure mechanisms under sneeze-induced stress condition in rats: a new animal model for evaluation of stress urinary incontinence.” American Journal of Physiology; Regulatory, Integrative and Comparative Physiology **285**(2): R356-65.

Kamo, I., Kaiho Y, et al. (2006). "Functional analysis of active urethral closure mechanisms under sneeze induced stress condition in a rat model of birth trauma." Journal Urology **176**(6 Pt 1): 2711-2715.

Kandabashi, T., Shimokawa H., et al. (2003). "Evidence for protein kinase C-mediated activation of Rho-kinase in a porcine model of coronary artery spasm. Arteriosclerosis, Thrombosis and Vascular Biology **23**(12): 2209-2214.

Kandasamy, K., Joseph K., et al. (2005). "Translational control of beta2-adrenergic receptor mRNA by T-cell-restricted intracellular antigen-related protein." Journal of Biological Chemistry **280**(3): 1931-1943.

Kaneko, T., Amano M., et al. (2000). "Identification of calponin as a novel substrate of Rho-kinase." Biochemical and Biophysical Research Communication **273**(1): 110-116.

Kang, S.H., Chess-Williams R., et al. (2013). "Induction of inflammatory cytokines and alteration of urothelial ATP, acetylcholine and prostaglandin E2 release by doxorubicin." European Journal of Pharmacology **700**(1-3): 102-109.

Kang, S.H., McDermott C., et al. (2015a). "Enhanced urothelial ATP release and contraction following intravesical treatment with the cytotoxic drug, doxorubicin." Naunyn-Schmiedebergs's Archives of Pharmacology **388**(7): 773-780.

Kang, S.H., Chess-Williams R., et al. (2015b). "Recovery of urothelial mediator release but prolonged elevations in interleukin-8 and nitric oxide secretion following mitomycin C treatment." Naunyn-Schmiedebergs's Archives of Pharmacology **388**(7): 781-791.

Kanyilmaz, S., Calis F.A., et al. (2013). "Bladder wall thickness and ultrasound estimated bladder weight in healthy adults with portative ultrasound device." Journal of Research in Medical Sciences **18**(2): 103-106.

Karam I., Droupy S., et al. (2005a). The precise location and nature of the nerves to the male human urethra: histological and immunohistochemical studies with three-dimensional reconstruction. European Urology **48**(5):858-864.

Karam, I., Moudouni S., et al. (2005b). "The structure and innervation of the male urethra: histological and immunohistochemical studies with three-dimensional reconstruction." Journal of Anatomy **206**(4): 395-403.

Karoor, V., & Malbon C. C. (1996). "Insulin-like growth factor receptor-1 stimulates phosphorylation of the β2-adrenergic receptor in vivo on sites distinct from those phosphorylated in response to insulin." Journal of Biological Chemistry **271**(46): 29347-29352.

Kato, M., Blanton R., et al. (2012). "Direct binding and regulation of RhoA protein by cyclic GMP-dependent protein kinase Ia." Journal of Biological Chemistry **287**(49): 41342-41351.

Keast, J.R. & Stephensen T.M. (2000). “Glutamate and aspartate immunoreactivity in dorsal root ganglion cells supplying visceral and somatic targets and evidence for peripheral axonal transport.” Journal of Comparative Neurology **424**(4): 577-587.

Kedia, G. T., offer, M., et al. (2013). “Pharmacologic characterization of human male urethral smooth muscle: an in vitro approach.” Urology **82**(6): 1451.e1413-e1459.

Keef, K.D., Saxton S.N., et al. (2013). “Functional role of vasoactive intestinal polypeptide in inhibitory motor innervation in the mouse internal anal sphincter.” Journal of Physiology **591**(6): 1489-1506.

Keffel, S., Alexandrov A., et al. (2000). “alpha(1)-adrenoceptor subtypes differentially couple to growth promotion and inhibition in Chinese hamster ovary cells.” Biochemical and Biophysical Research Communications **272**(3): 906-911.

Kelly, E., Bailey C. P., & Henderson G. (2008). “Agonist-selective mechanisms of GPCR desensitization.” British Journal of Pharmacology **153** (Suppl 1): S379-S388.

Kenton, K., Fitzgerald M.P. & Brubaker L. (2005). “Striated urethral sphincter activity does not alter urethral pressure during filling cystometry.” American Journal of Obstetrics and Gynecology **192**: 55–59.

Kenton, K., Mueller E. & Brubaker L. (2011). “Neuromuscular characterization of the urethra in continent women.” Femal Pelvic Medicine and Recontrcutive Surgery **17**(5)226-230.

Kenton, K., Lowenstein L., et al. (2007a). “Aging and overactive bladder may be associated with loss of urethral sensation in women.” Neurourology and Urodynamics **26**: 981–984.

Kenton, K., Simmons J., et al. (2007b). “Urethral and bladder current perception thresholds: normative data in women.” Journal of Urology **178**: 189–192.

Khandelwal, C., & Kistler C. (2013). “Diagnosis of urinary incontinence.” American Family Physician **87**(8): 543-550.

Khandelwal, P., Abraham S.N. & Apodaca G. (2009). “Cell biology and physiology of the uroepithelium.” American Journal of Physiology; Renal Physiology **297**(6); F1477–F1501.

Khasnis, M., Nakatomi A., et al. (2014). “Reconstituted human myosin light chain phosphatase reveals distinct roles of two inhibitory phosphorylation sites of the regulatory subunit, MYPT1.” Biochemistry **53**: 2701–2709.

Khurana, S., Chacon I. et al. (2004). "Vasodilatory effects of cholinergic agonists are greatly diminished in aorta from M3R-/- mice." European Journal of Pharmacology **493**(1-3):127-132.

Kibar, Y., Irkilata H.C., et al. (2011). “The effect of intravesical acetylsalicylic acid instillation on tissue prostaglandin levels after partial bladder outlet obstruction in rabbits.” Neurourology and Urodynamics **30**(8): 1646-1651.

Kim, Y.M. & Benovic J.L. (2002). “Differential roles of arrestin-2 interaction with clathrin and adaptor protein 2 in G protein-coupled receptor trafficking.” Journal of Biological Chemistry **277**(34): 30760-30768.

Kim, N., Cao W., et al. (2004). “Distinct kinases are involved in contraction of cat esophageal and lower esophageal sphincter smooth muscles.” American Journal of Physiology; Cell Physiology **287**(2): C384-C394.

Kim, R.J., Kerns J.M., et al. (2007). “Striated muscle and nerve fascicle distribution in the female rat urethral sphincter.” Anatomical Record (Hoboken) **290**(2): 145–154.

Kim, J.C., Yoo J.S., et al. (2008). “Muscarinic and purinergic receptor expression in the urothelium of rats with detrusor overactivity induced by bladder outlet obstruction.” British Journal of Urology International **101**(3): 371–375.

Kinugasa, Y., Arakawa T., et al. (2014). "Nerve supply to the internal anal sphincter differs from that to the distal rectum: an immunohistochemical study of cadavers." International Journal of Colorectal Disease **29**(4):429-436.

Kirby, A.C., Tan-Kim J. & Nager C.W. (2015). “Measurement of dynamic urethral pressures with a high-resolution manometry system in continent and incontinent women.” Female Pelvic Medicine and Recontructive Surgery **21**(2): 106-10.

Kirschstein, T., Protzel C., et al. (2014). “Age-dependent contribution of Rho kinase in carbachol-induced contraction of human detrusor smooth muscle in vitro.” Acta Pharmacologica Sinica **35**(1): 74-81.

Kirschstein, T., Sahre T., et al. (2015). “Inverse relationship of Rho kinase and myosin-light chain kinase expression in the aging human detrusor smooth muscle.” BMC Urology **15**(15): 104.

Kitazawa, T. & Somlyo A.P. (1990). “Desensitization and muscarinic re-sensitization of force and myosin light chain phosphorylation to cytoplasmic Ca2+ in smooth muscle.” Biochemical and biophysical research communication **172**(3): 1291-1297.

Kitazawa, T., Gaylinn B.D., et al. (1991). “G-protein-mediated Ca2+ sensitization of smooth muscle contraction through myosin light chain phosphorylation.” Journal of Biological Chemistry **266**(3): 1708-1715.

Kitazawa, T., Takizawa N., et al. (1999). “Reconstitution of protein kinase C-induced contractile Ca2+ sensitization in triton X-100-demembranated rabbit arterial smooth muscle” Journal of Physiology **520** (Pt 1): 139-152.

Kitazawa, T., Eto M., et al. (2000). “Agonist trigger G protein-mediated activation of the CPI-17 inhibitor phosphoprotein of myosin light chain phosphatase to enhance vascular smooth muscle contractility.” Journal of Biological and Chemistry **275**(14): 9897-9900.

Kitazawa, T., Eto M., et al. (2003). "Phosphorylation of the myosin phosphatase targeting subunit and CPI-17 during Ca^{2+} sensitization in rabbit smooth muscle." Journal of Physiology **546**(Pt 3): 879-889.

Kitazawa, T., Semba S., et al. (2009). "Nitric oxide-induced biphasic mechanism of vascular relaxation via dephosphorylation of CPI-17 and MYPT1." Journal of Physiology **587**(Pt 14): 3587-3603.

Kitta, T., Yoshikawa S., et al. (2016). "The effect of ovariectomy on urethral continence mechanisms during sneeze reflex in middle-aged versus young adult rats." Neurourology and Urodynamics **35**(1): 122-127.

Klarskov, N., Darekar A, et al. (2014). "Effect of fesoterodine on urethral closure function in women with stress urinary incontinence assessed by urethral pressure reflectometry." International Urogynecology Journal **25**(6): 755-760.

Klausner, A.P., Johnson C.M., et al. (2011). "Prostaglandin E2mediates spontaneous rhythmic contraction in rabbit detrusor muscle." Canadian Journal of Urology **18**(2): 5608-5614.

Klosterhalfen, B., Vogel P., et al. (1989). Topography of the inferior rectal artery: a possible cause of chronic, primary anal fissure. Diseases of the Colon and Rectum **32**(1): 43–52.

Kluck, P. (1980). "The autonomic innervation of the human urinary bladder, bladder neck and urethra: a histochemical study." The Anatomical Record **198**(3): 439-447.

Knepper, S. M., Buckner S. A., et al. (1995). "A-61603, a potent alpha 1-adrenergic receptor agonist, selective for the alpha 1A receptor subtype." Journal of Pharmacology and Experimental Therapeutics **274**(1): 97-103.

Kobayashi, K., Murata T., et al. (2011). "Prostaglandin E2-prostanoid EP3 signal induces vascular contraction via nPKC and ROCK activation in rat mesenteric artery." European Journal of Pharmacology **660**(2-3):375-380.

Kobayter, S., Young J.S. & Brain K.L. (2012). "Prostaglandin E2 induces spontaneous rhythmic activity in mouse urinary bladder independently of efferent nerves." British Journal of Pharmacology **165**(2): 401-413.

Koh, S.D., Dick G.M. & Sanders K.M. (1997). Small-conductance Ca(2+)-dependent K+ channels activated by ATP in murine colonic smooth muscle. American Journal of Physiology **273**(6 pt1): C2010–2021.

Koh, B.H., Roy R., et al. (2012). "Platelet-derived growth factor receptor-a cells in mouse urinary bladder: A new class of interstitial cells." Journal of Cellular and Molecular Medicine **16**(4): 691–700.

Kohler, T.S., Yadven M. et al. (2008). "The length of the male urethra." International Brazilian Journal of Urology **34**(4):451-454.

Kolta, M.G, Wallace L.J. (1984). “Age-related changes in sensitivity of rat urinary bladder to autonomic agents.” Mechanism of Ageing and Development **27**(2): 183-188.

Komari, S.O., Headley P.C., et al. (2013). “Evidence for a common mechanism for spontaneous rhythmic contraction and myogenic contraction induced by quick stretch in detrusor smooth muscle.” Physiological Reports **1**(6): e00168.

Komesu, Y.M., Schrader R.M., et al. (2016). “Epidemiology of mixed, stress, and urgency urinary incontinence in middle-aged/older women: the importance of incontinence history.” International Urogynecology Journal **27**(5): 763-772.

Kong, I.D., Koh S.D.& Sanders K.M. (2000). Purinergic activation of spontaneous transient outward currents in guinea pig taenia colonic myocytes. American Journal of Physiology; Cell Physiology **278**(2): C352–C362.

Kontani, H., Tsuji T. & Kimura S. (2000). “Effects of adrenergic alpha2-receptor agonists on urinary bladder contraction in conscious rats.” Japanese Journal of Pharmacology **84**(4): 381-390.

Korzick, D.H., Holiman D.A., et al. (2001). “Diminished alpha1-adrenergic-mediated contraction and translocation of PKC in senescent rat heart.” American journal of Physiology; Heart and Circulatory Physiology **281**(2): H581-H589.

Krasnopolsky, L., Levin R. M. & Longhurst P. A. (1995). “The role of the mucosa in the in vitro changes in guinea pig bladder function which occur after sensitization with ovalbumin.” Pharmacology **50**: 119-127.

Kreft, M.E., Romih R., et al. (2009). “Endocytotic activity of bladder superficial urothelial cells is inversely related to their differentiation stage.” Differentiation **77**(1): 48-59.

Kristiansson, P., Samuelsson E. et al. (2001). Reproductive hormones and stress urinary incontinence in pregnancy. Acta Onstetricia et Gynecologica Scandinavica **80**(12):1125-1130.

Krudewig, R., Langer B., et al. (2000). “Distinct internalization of M2 muscarinic acetylcholine receptors confers selective and long-lasting desensitization of signaling to phospholipase C.” Journal of Neurochemistry **74**(4): -1730.

Kubo, S., Doe I., et al. (2007). “Direct inhibition of endothelial nitric oxide synthase by hydrogen sulfide: contribution to dual modulation of vascular tension.” Toxicology **232**(1-2): 138-146.

Kubota, Y., Hashitani H., et al. (2008). "Altered distribution of interstitial cells in the guinea pig bladder following bladder outlet obstruction." Neurourology and urodynamics **27**(4):330-340.

Kullmann, F.A., Artim D.E., et al. (2008a). “Activation of muscarinic receptors in rat bladder sensory pathways alters reflex bladder activity.” Journal of Neuroscience **28**(8): 1977-1987.

Kullmann, F.A., Artim D., et al. (2008b). "Heterogeneity of muscarinic receptor-mediated Ca^{2+} responses in cultured urothelial cells from rat." The American Journal of Physiology; Renal Physiology **294**(4): F971–F981.

Kullmann, F.A., Limberg B.J., et al. (2009). "Effects of beta3-adrenergic receptor activation on rat urinary bladder hyperactivity induced by ovariectomy." Journal of Pharmacology and Experimental Therapeutics **330**: 704–717.

Kullmann, F.A., Downs T.R., et al. (2011). "Urothelial beta-3 adrenergic receptors in the rat bladder. Neurourology and Urodynamics **30**: 144–150.

Kumar, D., Waldron D., et al. (1990). Prolonged anorectal manometry and external anal sphincter electromyography in ambulant human subjects. Digestive Diseases and Sciences **35**(5): 641–648.

Kunit, T., Gratzke C., et al. (2014). "Inhibition of smooth muscle force generation by focal adhesion kinase inhibitors in the hyperplastic human prostate." American Journal of Physiology; Renal Physiology **307**(7): F823-F832.

Kunitake, H. & Poylin V. (2016). "Complications Following Anorectal Surgery." Clinics in Colon and Rectal Surgery **29**(1): 14-21.

Kuo, H.C., Lee K.S., et al. (2014). "Results of a randomized, double-blind, parallel-group, placebo- and active-controlled, multicenter study of mirabegron, a ß3-adrenoceptor agonist, in patients with overactive bladder in Asia." Neurourology and Urodynamics **34**(7): 685-692.

Kurian, N., Hall C.J., et al. (2009). "Full and partial agonists of muscarinic M3 receptors reveal single and oscillatory Ca2+ responses by beta 2-adrenoceptors." Journal of Pharmacology and Experimental Therapeutics **330**(2): 502-512.

Kurihara, R., Ishizu K., et al. (2016). "Study on Physiological Roles of Stimulation of Prostaglandin E2 Receptor Subtype EP2 in Urethral Function in Rats" Lower Urinary tract Symptoms **8**(2): 125-129.

Kurizaki, Y., Ishizuka O., et al. (2011). "Relation between expression of a(1)-adrenoceptor mRNAs in bladder mucosa and urodynamic findings in men with lower urinary tract symptoms." Scandinavian Journal of Nephrology **45**(1): 15-19.

Kyle, B.D., Bradley E., et al. (2013). "Mechanisms underlying activation of transient BK current in rabbit urethral smooth muscle cells and its modulation by IP3-generating agonists." American Journal of physiology; Cell Physiology **305**(6): C609-C622.

Lake, J.I. & Heuckeroth R.O. (2013). "Enteric nervous system development: migration, differentiation, and disease. American Journal of Physiology; Gastrointestinal and Liver Physiology **305**(1): G1-G24.

Lands, A.M, Arnold A., et al. (1967). "Differentiation of receptor systems activated by sympathomimetic amines." Nature **214**(5088): 597-598.

Lang, R.J., Hashitani H., et al. (2007a). "Spontaneous electrical and Ca2+ signals in typical and atypical smooth muscle cells and interstitial cell of Cajal-like cells of mouse renal pelvis." Journal of Physiology **583**(pt3): 1049-1068.

Lang, R.J., Hashitani H., et al. (2007b). "Role of Ca2+ entry and Ca2+ stores in atypical smooth muscle cell autorhythmicity in the mouse renal pelvis." British Journal of Pharmacology **152**(8): 1248-1259.

Laporte, S.A., Oakley R.H., et al. (2000). "The interaction of beta-arrestin with the AP-2 adaptor is required for the clustering of beta 2-adrenergic receptor into clathrin-coated pits." Journal of Biological Chemistry **275**(30): 23120-23126.

Larsson, B., Sjogren C., & Andersson K.E. (1986). "Regional distribution of α-adrenoceptor subtypes in the female rabbit urethra." Acta Physiologica Scandinavica **126**(1): 39-43.

Latifpour, J., Kondo S., et al. (1990). "Autonomic receptors in urinary tract: sex and age differences." Journal of Pharmacology and Experimental Therapeutics **253**(2): 661-667.

Lattion, A. L., Diviani D., & Cotecchia S. (1994). Truncation of the receptor carboxyl terminus impairs agonist-dependent phosphorylation and desensitization of the alpha 1B-adrenergic receptor. Journal of Biological Chemistry **269**(36): 22887-22893.

Lawrence, G.W., Aoki K.R. & Dolly J.O. (2010). "Excitatory cholinergic and purinergic signaling in bladder are equally susceptible to botulinum neurotoxin a consistent with co-release of transmitters from efferent fibers." Journal of Pharmacology and Experimental Therapeutics **334**(3): 1080-1086.

Lee, N.H. & Fraser C.M. (1993). "Cross-talk between m1 muscarinic acetylcholine and beta 2-adrenergic receptors. cAMP and the third intracellular loop of m1 muscarinic receptors confer heterologous regulation." Journal of Biological Chemistry **268**(11): 7949-7957.

Lee, H.Y., Bardini M. & Burnstock G. (2000). "Distribution of P2X receptors in the urinary bladder and the ureter of the rat." Journal of Urology **163**(6): 2002-2007.

Lee, K.B., Pals-Rylaarsdam R., et al. (1998). "Arrestin-independent internalization of the m1, m3, and m4 subtypes of muscarinic cholinergic receptors." Journal of Biological Chemistry **273**(21): 12967-1272.

Lee, T., Hedlund P., et al. (2007). "Urodynamic effects of a novel EP(1) receptor antagonist in normal rats and rats with bladder outlet obstruction." Journal of Urology **177**(4): 1562-1567.

Lee, Y.Y., Moujalled D., et al. (2013). "CREB-binding protein (CBP) regulates ß-adrenoceptor (ß-AR)-mediated apoptosis." Cell death and differentiation **20**(7): 941-952.

Lee, S.H., Cho S.T., et al. (2014). "Urinary incontinence in patients with Alzheimer's disease: relationship between symptom status and urodynamic diagnoses." International Journal of Urology **21**(7): 683-687.

Lei, B., Morris D.P., et al. (2009). “Lipid rafts constrain basal alpha(1A)-adrenergic receptor signaling by maintaining receptor in an inactive conformation.” Cell Signalling **21**(10): 1532-1539.

Leineweber, K., Klapproth S., et al. (2003). “Unchanged G-protein-coupled receptor kinase activity in the aging human heart.” Journal of the American College of Cardiology **42**(8): 1487-1492.

Leiria, L.O., Mónica F.Z., et al. (2011). “Functional, morphological and molecular characterization of bladder dysfunction in streptozotocin-induced diabetic mice: evidence of a role for L-type voltage-operated Ca2+ channels.” British Journal of Pharmacology **163**(6): 1276-1288.

Lentle, R.G., Reynolds G.W., et al. (2015). “Characterisation of the contractile dynamics of the resting ex vivo urinary bladder of the pig.” British Journal of Urology International **116**(6): 973-983.

Leone, A. M., Wiklund N. P., et al. (1994). “Release of nitric oxide by nerve stimulation in the human urogenital tract.” Neuroreport **5**(6): 733-736.

Lepor, H., Sunaryadi I., et al. (1992). "Quantitative morphometry of the adult human bladder." Journal of Urology **148**(2 pt 1):414-417.

Lepor, H., Tang R., et al. (1993). “Binding and functional properties of alpha1 adrenoceptors in different regions of the human prostate.” Journal of Urology **150**(1): 253-256.

Leung, M.W., Wong B.P., et al. (2006). Electrical stimulation and biofeedback exercise of pelvic floor muscle for children with faecal incontinence after surgery for anorectal malformation. Pediatric Surgery International **22**(12): 975-978.

Levin, R. M., Wein A. J., et al. (1995). “Effect of mucosal removal on the response of the feline bladder to pharmacological stimulation.” Journal of Urology **153**(4): 1291-1294.

Lewis, S.A. & Lewis J.R. (2006). “Kinetics of urothelial ATP release.” American Journal of Physiology; Renal Physiology **291**(2): F332-F340.

Li, A., Xi Q., et al. (2008). “Astrocyte-derived CO is a diffusible messenger that mediates glutamate-induced cerebral arteriolar dilation by activating smooth muscle Cell KCa channels.” Circulation Research **102**(2): 234-241.

Li, F., De Godoy M. & Rattan S. (2004). “Role of adenylate and guanylate cyclases in beta1-, beta2-, and beta3-adrenoceptor-mediated relaxation of internal anal sphincter smooth muscle.” Journal of Pharmacology and Experimental Therapeutics **308**(3): 1111-1120.

Li, L., Jiang C., et al. (2007). "Changes in T-type calcium channel and its subtypes in overactive detrusor of the rats with partial bladder outflow obstruction.” Neurourology and urodynamics **26**(6): 870-878.

Li, X.M., Yang H. et al. (2015). "Muscular structure at the male bladder outlet examined with successive celloidin-embedded slices." Urology **85**(3):629-635.

Liang, M., Eason M.G., et al. (1998). "Phosphorylation and functional desensitization of the alpha2A-adrenergic receptor by protein kinase C." Molecular Pharmacology **54**(1): 44-49.

Liang, M., Eason M. G., et al. (2002). "Phosphorylation of Ser360 in the third intracellular loop of the α2A-adrenoceptor during protein kinase C-mediated desensitization." European Journal of Pharmacology **437**(1-2): 41-46.

Liang, F.X., Bosland M.C., et al. (2005). "Cellular basis of urothelial squamous metaplasia: roles of lineage heterogeneity and cell replacement." Journal of Cell Biology **171**(5): 835-844.

Lies, B., Groneberg D. & Friebe A. (2014). "Toward a better understanding of gastrointestinal nitrergic neuromuscular transmission." Neurogastroenetrology and Motility **26**(7): 901-912.

Lilja, H. & Abrahamsson P.A. (1988). "Three predominant proteins secreted by the human prostate gland." Prostate **12**(1): 29-38.

Lim, S.H., Wang T.J., et al. (2013). "The distribution of muscles fibers and their types in the female rat urethra: cytoarchitecture and three-dimensional reconstruction." Anatomical Record **296**(10): 1640-1649.

Limberg, B.J., Andersson K.E., et al. (2010). "b-Adrenergic receptor subtype expression in myocyte and non-myocyte cells in human female bladder." Cell and Tissue research **342**(2): 295-306.

Lin, A.T., Sun M.J., et al. (2008). "Duloxetine versus placebo for the treatment of women with stress predominant urinary incontinence in Taiwan: a double-blind, randomized, placebo-controlled trial." BMC Urology **8**: 2.

Lin, R., Degan S., et al. (2012). "Chronic treatment in vivo with β-adrenoceptor agonists induces dysfunction of airway β(2) -adrenoceptors and exacerbates lung inflammation in mice." British Journal of Pharmacology 165(7): 2365-2377.

Linden, D.R., Sha L., et al. (2008). "Production of the gaseous signal molecule hydrogen sulfide in mouse tissues." Journal of Neurochemistry **106**(4): 1577-1585.

Lino, S., Horiguchi K., et al. (2009). Interstitial cells of Cajal contain signalling molecules for transduction of nitrergic stimulation in guinea pig caecum. Neurogastroenterology and Motility **21**(5):542-550, e12-e13.

Lips, K.S., Wunsch J., et al. (2007). "Acetylcholine and molecular components of its synthesis and release machinery in the urothelium." European Urology **51**(4): 1042-1053.

Liu, B., Wang L., et al. (2014). "Prevalence and risk factors of urinary incontinence among Chinese women in Shanghai." International Journal of Clinical and Experimental Medicine **7**(3): 686-696.

Liu, Q.Y., Karpinski E. & Pang P.K. (1994). "The L-type calcium channel current is increased by alpha-1 adrenoceptor activation in neonatal rat ventricular cells." The Journal of Pharmacology and Experimental Therapeutics **271**(2): 935-943.

Lluel, P., Palea S., et al. (2000). "Functional and morphological modifications of the urinary bladder in aging female rats." American Journal of Physiology: Regulatory, Integrative and Comparative Physiology **278**(4): R964-R972.

Lluel, P., Deplanne V., et al. (2003). Age-related changes in urethrovesical coordination in male rats: relationship with bladder instability? American Journal of Physiology; Regulatory, Integrative and Comparative Physiology **284**(5): R1287–R1295.

Logadottir, Y.R., Ehren I., et al. (2004). "Intravesical nitric oxide production discriminates between classic and nonulcer interstitial cystitis." Journal of Urology **171**(3): 1148-1150; discussion 50-1.

Lohse, M. J., Andexinger S., et al. (1992). "Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the beta 2-adrenergic receptor and rhodopsin systems." Journal of Biological Chemistry **267**(12): 8558-8564.

Lomasney, J. W., Cotecchia, S., et al. (1991). Molecular cloning and expression of the cDNA for the a1Aadrenergic receptor. Journal of Biological Chemistry **266** : 6365-6369.

Longhurst, P. A., Eika B., et al. (1992). "Comparison of urinary bladder function in 6 and 24 month male and female rats." Journal of Urology **148**(5): 1615-1620.

Lopes, G.S., Ferreira A.T., et al. (2006). "Aging-related changes of intracellular Ca^{2+} stores and contractile response of intestinal smooth muscle." Experimental Gerontology **41**(1): 55-62.

Lorenzi, B., Brading A.F. & Mortensen N.J. (2014). "Interstitial cells of Cajal modulate the tone of the human internal anal sphincter in vitro." Diseases of the Colon and Rectum **57**(3): 370-377.

Lu, L., Sirish P., et al. (2015). "Regulation of gene transcription by voltage-gated L-type calcium channel, Cav1.3." Journal of Biological Chemistry 290(8): 4663-4676.

Lubowski, D.Z., Nicholls R.J. (1988). Internal anal sphincter in neurogenic fecal incontinence. Gastroenterology **95**(4): C997–C1002.

Lund, J.N (2006). "Nitric oxide deficiency in the internal anal sphincter of patients with chronic anal fissure." International Journal of Colorectal Disease **21**(7): 673-675.

Lunniss, P.J., Gladman M.A., et al. (2004). "Risk factors in acquired faecal incontinence. Journal of the Royal Society of Medicine **97**(3): 111-6.

Luo, D.Y., Wazir R., et al. (2013). "Integrin av mediates contractility whereas integrin a4 regulates proliferation of human bladder smooth muscle cells via FAK pathway under physiological stretch." Journal of Urology **190**(4): 1421-1429.

Lymperopoulos, A., Rengo G., et al. (2007). "Adrenal GRK2 upregulation mediates sympathetic overdrive in heart failure." Nature Medicine **13**(3): 315-323.

Lyons, A.D., Gardiner T.A. & McCloskey K.D. (2007). "Kit-positive interstitial cells in the rabbit urethra: structural relationships with nerves and smooth muscle." British Journal of Urology International **99**(3): 687-694.

Ma, X., Zhao Y., et al. (2012). "Acute activation of ß2-adrenergic receptor regulates focal adhesions through ßArrestin2- and p115RhoGEF protein-mediated activation of RhoA." Journal of Biological Chemistry **287**(23): 18925-18936.

Macia, E., Partisani M., et al. (2012). "Arf6 negatively controls the rapid recycling of the ß2 adrenergic receptor." Journal of Cell Science **125**(Pt 17): 4026-35.

Macmillan, A.K., Merrie A.E., et al. (2004). "The prevalence of fecal incontinence in community-dwelling adults: a systematic review of the literature." Diseases of the Colon and Rectum **47**(8): 1341-1349.

Madalinski, M.H. (2011). "Identifying the best therapy for chronic anal fissure." World Journal of Gastrointestinal Pharmacology and Therapeutics **2**(2): 9-16.

Magalhaes, A.C., Holmes K.D., et al. (2010). "CRF receptor 1 regulates anxiety behavior via sensitization of 5-HT2 receptor signaling." Nature Neuroscience **13**(5): 622-629.

Maggi, C. A., Santicioli P., et al. (1987). "The presence of mucosa reduces the contractile response of the guinea-pig urinary bladder to substance P." The Journal of Pharmacy and Pharmacology **39** (8): 653-655.

Mahony, R.T., Malone P.A., et al. (2004). Randomized clinical trial of intra-anal electromyographic biofeedback physiotherapy with intra-anal electromyographic biofeedback augmented with electrical stimulation of the anal sphincter in the early treatment of postpartum fecal incontinence. American Journal of Obstetrics and Gynecology **191**(3): 885-890.

Malmqvist, U., Hedlund P., et al. (2004). "Female pig urethral tone is dependent on Rho guanosine triphosphatases and Rho-associated kinase." Journal of Urology **171**(5): 1955-1958.

Malouf, A.J., Vaizey C.J., et al. (2001). "Internal anal sphincter augmentation for fecal incontinence using injectable silicone biomaterial." Diseases of the Colon and Anorectum **44**(4): 595-600.

Manara, L., Croci T., et al. (2000). "Functional assessment of beta adrenoceptor subtypes in human colonic circular and longitudinal (taenia coli) smooth muscle." Gut **47**(3): 337-342.

Mangera, A. Osman N.I. & Chapple C.R. (2013). "Anatomy of the lower urinary tract." Surgery **31**(7): 319–325.

Mansfield, K.J., Liu L., et al. (2005). "Muscarinic receptor subtypes in human bladder detrusor and mucosa, studied by radioligand binding and quantitative competitive RT-PCR: changes in ageing." British Journal of Pharmacology **144**(8): 1089-1099.

Mapel, D.W., Schum M. & Von Worley A. (2014). "The epidemiology and treatment of anal fissures in a population-based cohort." BMC Gastroenterology **14**: 129.

Markland, A.D., Richter H.E. et al., (2011). "Prevalence and trends of urinary incontinence in adults in the United States, 2001 to 2008." Journal of Urology **186** (2): 589-593.

Markland, A.D., Burgio K.L. et al. (2015). Loperamide Versus Psyllium Fiber for Treatment of Fecal Incontinence: The Fecal Incontinence Prescription (Rx) Management (FIRM) Randomized Clinical Trial. Diseases of the Colon and Rectum **58**(10): 983-993.

Markland, A.D., Dunivan G.C., et al. (2016). "Anal Intercourse and Fecal Incontinence: Evidence from the 2009-2010 National Health and Nutrition Examination Survey." American journal of Gastroeneterology **111**(2): 269-274.

Marquetant, R., Brehm B. & Strasser R.H. (1992). "Chronic β-blockade transregulates inhibitory A1 adenosine and muscarinic M2 receptors of the adenylyl cyclase system." Journal of Molecular and Cellular Cardiology **24** (5): 535–548.

Martin, G.R., McKnight G.W, et al. (2010). "Hydrogen sulphide synthesis in the rat and mouse gastrointestinal tract." Digestive and Liver Diseases **42**(2): 103-109.

Martinsen, A., Schakman O., et al. (2014). "Myosin light chain kinase controls voltage-dependent calcium channels in vascular smooth muscle." Pflugers Archives; European Journal of Physiology **466**(7): 1377-1389.

Masunaga, K., Yoshida M., et al. (2006). "Prostaglandin E2 release from isolated bladder strips in rats with spinal cord injury. International Journal of Urology **13**(3): 271-276.

Masunaga, K., Chapple C. R., et al. (2010). "The β3-adrenoceptor mediates the inhibitory effects of β-adrenoceptor agonists via the urothelium in pig bladder dome." Neurourology and Urodynamics **29**(7:1320-1325.

Matsui, M., Griffin M.T., et al. (2003). "Increased relaxant action of forskolin and isoproterenol against muscarinic agonist-induced contractions in smooth muscle from M2 receptor knockout mice." Journal of Pharmacology and Experimental Therapeutics **305**: 106–113.

Mattiasson, A., Andersson K. E., & Sjogren C. (1984). “Adrenoceptors and cholinoceptors controlling noradrenaline release from adrenergic nerves in the urethra of rabbit and man.” Journal of Urology **131**(6): 1190-1195.

Maureira, A., Sánchez R., et al. (2016). “The CREB Transcription Factor Controls Transcriptional Activity of the Human RIC8B Gene.” Journal of Cellular Biochemistry **117**(8): 1797-1805.

Mawby, D.I., Meric S.M. et al. (1991). "Pharmacological relaxation of the urethra in male cats: a study of the effects of phenoxybenzamine, diazepam, nifedipine and xylazine." Canadian Journal of Veterinary Research **55**(1):28-32.

Mazzuoli, G. & Schemann M. (2009). “Multifunctional rapidly adapting mechanosensitive enteric neurons (RAMEN) in the myenteric plexus of the guinea pig ileum.” Journal of Physiology **587**(Pt 19): 4681-4694.

Mazzuoli, G. & Schemann M. (2012). “Mechanosensitive enteric neurons in the myenteric plexus of the mouse intestine.” Plos One **7**(7): e39887.

Mc Kinley, M. P., & O'loughlin V. D. (2008). “Human anatomy.” From McGraw-Hill higher education.

McCafferty, G.P., Misajet B.A., et al. (2008) “Enhanced bladder capacity and reduced prostaglandin E2-mediated bladder hyperactivity in EP3 receptor knockout mice.” American Journal of Physiology; Renal Physiology **295**(2): F507-F514.

McCloskey, K.D. & Gurney A.M. (2002). “Kit positive cells in the guinea pig bladder.” Journal of Urology **168** (2): 832-836.

McCloskey, K.D. (2010). “Interstitial cells in the urinary bladder--localization and function.” Neurourology and Urodynamics **29**(1): 82-87.

McDermott, C., Chess-Williams R., et al. (2012). “Effects of Pseudomonas aeruginosa virulence factor pyocyanin on human urothelial cell function and viability.” Journal of Urology **187**(3): 1087-1093.

McDermott, C., Chess-Williams R., et al. (2013). “Alterations in acetylcholine, PGE2 and IL6 release from urothelial cells following treatment with pyocyanin and lipopolysaccharide.” Toxicology In Vitro **27**(6): 1693-1698.

McDonnell, B., Hamilton R., et al. (2008). “Functional evidence for purinergic inhibitory neuromuscular transmission in the mouse internal anal sphincter.” American Journal of Physiology; Gastrointestinal and liver Physiology **294**(4): G1041-G1051.

McGee, M.J. & Grill W.M. (2014). “Selective co-stimulation of pudendal afferents enhances bladder activation and improves voiding efficiency.” Neurourology and Urodynamics **33**(8): 1272-1278.

McGraw, D. W., Donnelly E. T., et al. (1998). “Role of βARK in Long-Term Agonist-Promoted Desensitisation of the β2-Adrenergic Receptor.” Cellular Signalling **10**(3): 197-204.

McHale, N. G., Hollywood M. A., et al. (2006). “Organization and function of ICC in the urinary tract.” Journal of Physiology **576**(Pt 3)**:** 689-694.

McKeag, N.A., Thornbury K.D., et al. (2001). “Role of calcium-activated chloride channels in myogenic tone in the rat urethra.” Journal of Physiology **536**: 93P.

McKenzie, S., Watson T., et al. (2016). “Stress urinary incontinence is highly prevalent in recreationally active women attending gyms or exercise classes.” International Urogynaecology Journal **27**(8): 1175-1184.

McLatchie, L.M. & Fry C.H. (2015). “ATP release from freshly isolated guinea-pig bladder urothelial cells: a quantification and study of the mechanisms involved.” British Journal of Urology International 115(6): 987-993.

McLatchie, L.M., Young J.S. & Fry C.H. (2014). “Regulation of ACh release from guinea pig bladder urothelial cells: potential role in bladder filling sensations.” British Journal of Pharmacology **171**(14): 3394-3403.

McMurray, G., Dass N. & Brading A.F. (1998). “Purinoceptor subtypes mediating contraction and relaxation of marmoset urinary bladder smooth muscle.” British Journal of Pharmacology **123**(8): 1579-1586.

McPherson, J., Rivero G. et al. (2010). "μ-opioid receptors: correlation of agonist efficacy for signalling with ability to activate internalization." Molecular Pharmacology **74**(4):756-766.

Medina, C.A., Costantini E., et al. (2016). “Evaluation and surgery for stress urinary incontinence: A FIGO working group report.” Neurourology and Urodynamics (in print).

Medina, L.C., Vázquez-Prado J., et al. (1998). “Crosstalk: phosphorylation of alpha1b-adrenoceptors induced through activation of bradykinin B2 receptors.” FEBS Letter **422**(2): 141-145.

Melville, J.L., Delaney K., et al. (2005). “Incontinence severity and major depression in incontinent women.” Obstetrics and Gynecology **106**(3): 585-592.

Merlet, P., Dubois-Randé J.L., et al. (1992). “Myocardial beta-adrenergic desensitization and neuronal norepinephrine uptake function in idiopathic dilated cardiomyopathy.” Journal of Cardiovascular Pharmacology **19**(1): 10-16.

Merrill, L., Gonzalez E.J., et al. (2016). “Receptors, channels, and signalling in the urothelial sensory system in the bladder.” Nature reviews, Urology. **13**(4): 193-204.

Meyer, I. & Richter H.E. (2016). “Evidence-Based Update on Treatments of Fecal Incontinence in Women.” Obstetrics and Gynecology Clinics in North America **43**(1): 93-119.

Michel, M.C. (2014). “Do ß-adrenoceptor agonists induce homologous or heterologous desensitization in rat urinary bladder?” Naunyn-Schmiedeberg's Archives of Pharmacology. **387**(3): 215-224.

Michel, M. C., & Vrydag W. (2006). “Alpha1-, alpha2- and beta-adrenoceptors in the urinary bladder, urethra and prostate.” British Journal of Pharmacology **147** Suppl 2: S88-119.

Michel, M.C. & Barendrecht M.M. (2008). “Physiological and pathological regulation of the autonomic control of urinary bladder contractility.” Pharmacology and Therapeutics **117**(3): 297-312.

Michel, M.C., Ochodnicky P., et al. (2011). “b-Adrenoceptor agonist effects in experimental models of bladder dysfunction.” Pharmacology & Therapeutics **131**; 40–49.

Michels, L., Blok B.F. et al. (2015). "Supraspinal Control of Urine Storage and Micturition in Men--An fMRI Study." Cerebral Cortex **25**(10):339-3380.

Michel-Reher, M.B. & Michel M.C (2013). “Agonist-induced desensitization of human ß3-adrenoceptors expressed in human embryonic kidney cells.” Naunyn-Schmiedeberg’s Archive of Pharmacology **386**: 843–851.

Millard, R.J., Moore K., et al. (2004). “Duloxetine vs placebo in the treatment of stress urinary incontinence: a four-continent randomized clinical trial.” British Journal of Pharmacology International **93**(3): 311-318.

Miller, R., Lewis G.T., et al. (1988a). Sensory discrimination and dynamic activity in the anorectum: evidence using a new ambulatory technique. British Journal of Surgery **75**(10): 1003–1007.

Miller, R., Bartolo D.C., et al. (1988b). Anorectal sampling: a comparison of normal and incontinent patients. British Journal of Surgery 75(1): 44–47.

Mills, K.A., Hausman N., & Chess-Williams R. (2008). “Characterization of the α 1-adrenoceptor subtype mediating contractions of the pig internal anal sphincter.” British Journal of Pharmacology **155**(1): 110-117.

Minneman, K. P., Theroux T. L., et al. (1994). “Selectivity of agonists for cloned alpha 1-adrenergic receptor subtypes.” Molecular Pharmacology **46**(5): 929-936.

Miyazato, M., Kaiho Y., et al. (2008). “Effect of duloxetine, a norepinephrine and serotonin reuptake inhibitor, on sneeze-induced urethral continence reflex in rats.” American Journal of Physiology; Renal Physiology **295**(1): F264-F271.

Miyoshi, K., Kawakami N., et al. (2007). “Heterologous up-regulation of the histamine H1 receptor by M3 muscarinic receptor-mediated activation of H1-receptor gene transcription.” Journal of Pharmacy and Pharmacology **59**(6): 843-848.

Mizuno, Y., Isotani E., et al. (2008). "Myosin light chain kinase activation and calcium sensitization in smooth muscle in vivo." American Journal of Physiology; cell Physiology **295**(2): C358-C364.

Molina-Muñoz, T., Romero-Avila M.T. & García-Sáinz J.A. (2006). "Insulin-like growth factor-I induces alpha (1B)-adrenergic receptor phosphorylation through G beta gamma and epidermal growth factor receptor transactivation." Molecular Endocrinology **20**(11): 2773-2783.

Molina-Muñoz, T., Romero-Avila M.T., et al. (2008). "Phosphorylation, desensitization and internalization of human alpha1B-adrenoceptors induced by insulin-like growth factor-I." European Journal of Pharmacology **578**(1): 1-10.

Monaghan, K.P., Johnston L. & McCloskey K.D. (2012). "Identification of PDGFRa positive populations of interstitial cells in human and guinea pig bladders." Journal of Urology **188**: 639–47.

Montiel, M., Quesada J., & Jiménez E. (2004). "Activation of second messenger-dependent protein kinases induces muscarinic acetylcholine receptor desensitization in rat thyroid epithelial cells." Molecular and Cellular Endocrinology **223**(1–2): 35-41.

Moore, R.H., Tuffaha A., et al. (1999). "Agonist-induced sorting of human beta2-adrenergic receptors to lysosomes during downregulation." Journal of Cell Sciences **112**(pt3): 329-338.

Moore, R.H., Millman E.E., et al. (2007). "Salmeterol stimulation dissociates beta2-adrenergic receptor phosphorylation and internalization." American Journal of Respiratory Cell and Molecular Biology **36**(2): 254-261.

Morishima, S., Tanaka T., et al. (2007). "Identification of alpha-1L and alpha-1A adrenoceptors in human prostate by tissue segment binding." Journal of Urology **177**(1): 377-381.

Morishima, S., Suzuki F., et al. (2008). "Identification of the alpha1L-adrenoceptor in rat cerebral cortex and possible relationship between alpha1L and alpha1A-adrenoceptors." British Journal of Pharmacology **153**(7): 1485-1494.

Morita, T., Ando M., et al. (1994). "Effects of prostaglandins E1, E2 and F2 alpha on contractility and cAMP and cGMP contents in lower urinary tract smooth muscle." Urology International **52**(4): 200-203.

Morita, T., Iizuka H., et al. (2000). "Function and distribution of beta3-adrenoceptors in rat, rabbit and human urinary bladder and external urethral sphincter. Journal of Smooth muscle research **36**(1): 21-32.

Moro, C., Tajouri L., & Chess-Williams R. (2013). "Adrenoceptor function and expression in bladder urothelium and lamina propria. Journal of Urology **81**(1): 211; e1-e7.

Morris, D.P., Price R.R., et al. (2004). "Cellular trafficking of human alpha1a-adrenergic receptors is continuous and primarily agonist-independent." Molecular Pharmacology **66**(4): 843-854.

Morris, D.P., Lei B., et al. (2008). "The alpha1a-adrenergic receptor occupies membrane rafts with its G protein effectors but internalizes via clathrin-coated pits." Journal of Biological Chemistry **283**(5): 2973-2985.

Morrison, J. (1999). "The activation of bladder wall afferent nerves." Experimental Physiology **84** (1): 131-136.

Morrison, K.J., Moore R.H., et al. (1996). "Repetitive endocytosis and recycling of the beta 2-adrenergic receptor during agonist-induced steady state redistribution." Molecular Pharmacology **50**(3): 692-699.

Mosser, V.A., Jones K.T., et al. (2008). "Differential role of beta-arrestin ubiquitination in agonist-promoted down-regulation of M1 vs M2 muscarinic acetylcholine receptors." Journal of Molecular Signallig **3**: 20.

Moszkowicz, D., Peschaud F., et al. (2012). "Internal anal sphincter parasympathetic-nitrergic and sympathetic-adrenergic innervation: a 3-dimensional morphological and functional analysis." Diseases of the Colon and Rectum **55**(4): 473-481.

Motomura, S., Deighton N.M., et al. (1990). "Chronic β1-adrenoceptor antagonist treatment sensitizes β2-adrenoceptors, but desensitizes M2-muscarinic receptors in the human right atrium." British Journal of Pharmacology **101** (2): 363–369.

Mulvey, M.A., Lopez-Boado Y.S., et al. (1998). "Induction and evasion of host defenses by type 1-piliated uropathogenic Escherichia coli." Science **282**(5393): 1494-1497.

Mundey, M.K., Jonas M., et al. (2000). "Pharmacological characterization of neurogenic responses of the sheep isolated internal anal sphincter." British Journal of Pharmacology **130**(3): 489-494.

Munoz, A., Smith C.P., et al. (2011). "Overactive and underactive bladder dysfunction is reflected by alterations in urothelial ATP and NO release." Neurochemistry International **58**(3): 295-300.

Munoz, A., Somogyi G.T., et al. (2012). "Modulation of bladder afferent signals in normal and spinal cord-injured rats by purinergic P2X3 and P2X2/3 receptors." British Journal of Urology International **110**(8 pt b): E409-E414.

Muntener, M., Schurch B., et al. (2006). "Systemic nitric oxide augmentation leads to a rapid decrease of the bladder outlet resistance in healthy men." European Urology **50**(1): 112-7; discussion 117-118.

Murakami, S., Chapple C.R., et al. (2007). "The role of the urothelium in mediating bladder responses to isoprenaline." British Journal of Urology International **99**(3): 669-673.

Muramatsu, I., Murata S., et al. (1998). "Alpha1-adrenoceptor subtypes and two receptor systems in vascular tissues." Life Sciences **62**(17-18): 1461-1465.

Muramatsu, I., Morishima S., et al. (2008). "Identification of alpha 1L-adrenoceptor in mice and its abolition by alpha 1A-adrenoceptor gene knockout." British Journal of Pharmacology **155**(8): 1224-1234.

Muramatsu, I., Suzuki F., et al. (2009). "Expression of distinct alpha 1-adrenoceptor phenotypes in the iris of pigmented and albino rabbits." British Journal of Pharmacology **158**(1): 354-360.

Murányi, A., Derkach D., et al. (2005). "Phosphorylation of Thr695 and Thr850 on the myosin phosphatase target subunit: inhibitory effects and occurrence in A7r5 cells." FEBS Letters **579**(29): 6611-6615.

Murata, S., Taniguchi T. & Muramatsu I. (1999). Pharmacological analysis of the novel, selective alpha1-adrenoceptor antagonist, KMD-3213, and its suitability as a tritiated radioligand. British Journal of Pharmacology **127**(1): 19–26.

Musselman, D.M., Ford A.P., et al. (2004). "A randomized crossover study to evaluate Ro 115-1240, a selective alpha1A/1L-adrenoceptor partial agonist in women with stress urinary incontinence." British Journal of Urology international **93**(1): 78-83.

Mutoh, S., Latifpour, J., et al. (1997). "Evidence for the presence of regional differences in the subtype specificity of muscarinic receptors in rabbit lower urinary tract." Journal of Urology **157**(2): 717-721.

Na, Y.J., Guo Y.L. & Gu F.L. (1998). Clinical comparison of selective and non-selective alpha 1A-adrenoceptor antagonists for bladder outlet obstruction associated with benign prostatic hyperplasia: studies on tamsulosin and terazosin in Chinese patients. The Chinese Tamsulosin Study Group. Journal of Medicine **29**(5-6): 289-304.

Na, H.R., Park M.H., et al. (2015). "Urinary incontinence in Alzheimer's disease is associated with Clinical Dementia Rating-Sum of Boxes and Barthel Activities of Daily Living." Asia-Pacific Psychiatry **7**(1): 113-120.

Naga Prasad, S.V., Laporte S.A., et al. (2002). "Phosphoinositide 3-kinase regulates beta2-adrenergic receptor endocytosis by AP-2 recruitment to the receptor/beta-arrestin complex." Journal of Cell Biology **158**(3): 563-575.

Nagahama, K., Tsujii T., et al. (1998). "Differences between proximal and distal portions of the male rabbit posterior urethra in the physiological role of muscarinic cholinergic receptors." British Journal of Pharmacology **124**(6): 1175-1180.

Naik, J.S. & Walker B.R. (2003). "Heme oxygenase-mediated vasodilation involves vascular smooth muscle cell hyperpolarization." American Journal of Physiology; Heart and circulatory Physiology **285**(1): H220-H228.

Nakagawa, T., Misawa H., et al. (2005). “Absence of peristalsis in the ileum of W/W(V) mutant mice that are selectively deficient in myenteric interstitial cells of Cajal.” Journal of Smooth Muscle Research **41**(3): 141-151.

Nakanishi, K., Kamai T., et al. (2009). “Expression of RhoA mRNA and activated RhoA in urothelium and smooth muscle, and effects of a Rho-kinase inhibitor on contraction of the porcine urinary bladder.” Neurourology and Urodynamics **28**(6): 521-528.

Nalli, A.D., Rajagopal S., et al. (2015). “Inhibition of RhoA-dependent pathway and contraction by endogenous hydrogen sulfide in rabbit gastric smooth muscle cells.” American Journal of Physiology; Cell physiology **308**(6): C485-C495.

Nandigama, R., Bonitz M., et al. (2010). “Muscarinic acetylcholine receptor subtypes expressed by mouse bladder afferent neurons.” Neuroscience **168**(3): 842-850.

Nanoff, C., Ströher M., et al. (1990). “Desensitization pattern of cardiac beta-adrenoceptor subtypes by prolonged in vivo infusion of pindolol and celiprolol in rats.” Basic Research in Cardiology **85**(1): 88-95.

Nantel, F., Bonin H., et al. (1993). “The human ß3-adrenergic receptor is resistant to short term agonist-promoted desensitization.” Molecular Pharmacology **43**(4): 548–555.

Nantel, F., Bouvier M., et al. (1995). “Functional effects of long-term activation of human ß2-and ß3-adrenoceptor signalling.” British Journal of Pharmacology **114**(5): 1045–1051.

Narayanan, D., Adebiyi A. & Jaggar J.H. (2012). “Inositol trisphosphate receptors in smooth muscle cells.” American Journal of Physiology; Heart and Circulatory Physiology **302**(11): H2190-H2210.

Nasu, K., Moriyama N., et al. (1998). “Quantification and distribution of alpha1-adrenoceptor subtype mRNAs in human proximal urethra.” British Journal of Pharmacology **123**(7): 1289-1293.

Negoro, H., Lutz S.E., et al. (2013). “Pannexin 1 involvement in bladder dysfunction in a multiple sclerosis model.” Scientific Reports **3**: 2152.

Nelson, R. (2006). “Non surgical therapy for anal fissure.” Cochrane database of systematic reviews **4**: CD003431.

Nelson, R.L., Thomas K., et al. (2012). “Non surgical therapy for anal fissure. Cochrane database of systematic reviews **2**: CD003431.

Ng, K.S., Nassar N., et al. (2015). "Prevalence of functional bowel disorders and faecal incontinence: an Australian primary care survey." Colorectal Diseases **17**(2):150-159.

Nile, C.J., de Vente J. & Gillespie J.I. (2010). “Stretch independent regulation of prostaglandin E(2) production within the isolated guinea-pig lamina propria.” British Journal of Urology International **105**(4):540-548.

Nile, C.J. & Gillespie J.I. (2012). “Interactions between cholinergic and prostaglandin signaling elements in the urothelium: role for muscarinic type 2 receptors.” Urology **79**(1): 240–e23.

Nilvebrant, L., Andersson K.E., et al. (1997). “Tolterodine--a new bladder-selective antimuscarinic agent.” European Journal of Pharamcology **327**(2-3): 195-207.

Nisar, P.J., Gruss H.J., et al. (2005). “Intra-anal and rectal application of L-erythro methoxamine gel increases anal resting pressure in healthy volunteers.” British Journal of Surgery **92**(12): 1539-1545.

Nisar P.J., Gruss H.J. et al. (2007). "Intra-anal application of L-erythro methoxamine gel increases anal resting pressure in patients with incontinence." British Journal of Surgery **94**(9):1155-1161.

Nishimune, A., Suzuki F., et al. (2010a). “Alpha 1-adrenoceptor pharmacome: alpha 1L-adrenoceptor and alpha 1A-adrenoceptor in the lower urinary tract.” International Journal of Urology **17**(1): 31-37.

Nishimune, A., Suzuki F., et al. (2010b). “Identification of cysteine-rich epidermal growth factor-like domain 1alpha (CRELD1alpha) as a novel alpha1A-adrenoceptor-down-regulating protein and establishment of an alpha1L-adrenoceptor-expressing cell line.” Journal of Pharmacological Sciences **113**(2): 169-181.

Nishimune, A., Yoshiki H., et al. (2012). “Phenotype pharmacology of lower urinary tract α 1- adrenoceptors.” British Journal of Pharmacology **165**(5): 1226-1234.

Nishizaki, T., & Sumikawa K. (1998). “Effects of PKC and PKA phosphorylation on desensitization of nicotinic acetylcholine receptors.” Brain Research **812**(1–2): 242-245.

Nissan, S., Vinograd Y., et al. (1984). “Physiological and pharmacological studies of the internal anal sphincter in the rat.” Journal of Pediatric Surgery **19**(1): 12-14.

Nitti, V.W., Auerbach S. et al. (2013). "Results of a randomized phase III trial of mirabegron in patients with overactive bladder." Journal of Urology **189**(4):1388-1395.

Nitti, V.W., Chapple C.R., et al. (2014). “Safety and tolerability of the ß3 -adrenoceptor agonist mirabegron, for the treatment of overactive bladder: results of a prospective pooled analysis of three 12-week randomised Phase III trials and of a 1-year randomised Phase III trial.” International Journal of clinical practice **68**(8): 972-985.

Nobles, K.N., Xiao K., et al. (2011). “Distinct phosphorylation sites on the β(2)-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin.” Science Signaling **4**(185): ra51.

Noel, S., Cambier C., et al. (2010). “Combined pharmacokinetic and urodynamic study of the effects of oral administration of phenylpropanolamine in female Beagle dogs.” Veterinary Journal **184**(2): 201-207.

Noel, S., Massart L. & Hamaide A. (2013). "Urodynamic investigation by telemetry in Beagle dogs: validation and effects of oral administration of current urological drugs: a pilot study." BMC Veterinary Research **9**: 197.

Nomiya, M. & Yamaguchi O. (2003). "A quantitative analysis of mRNA expression of alpha 1 and beta-adrenoceptor subtypes and their functional roles in human normal and obstructed bladders." Journal of Urology **170**(2 pt1): 649-653.

Noto, H., Roppolo J.R. et al., (1991). "Electrophysiological analysis of the ascending and descending components of the micturition reflex pathway in the rat." Brain Research **549**(1): 95-105.

Nzimbala, M.J. & Bruyninx L. (2007). "Chronic anal fissure from suspected adult sexual abuse in a traumatic anal sex practice patient." Acta Chirirgica Belgica **107**(5): 566-569.

O'Kelly, T.J., Brading A. & Mortensen N.J. (1993a). "In vitro response of the human anal canal longitudinal muscle layer to cholinergic and adrenergic stimulation: evidence of sphincter specialization." British Journal of Surgery **80**(10): 1337-1341.

O'Kelly, T., Brading A. & Mortensen N. (1993b). "Nerve mediated relaxation of the human internal anal sphincter: the role of nitric oxide." Gut **34**(5): 689-693.

O'Neill, A.B., Buckner S.A., et al. (2001). "Pharmacological properties of A-204176, a novel and selective alpha1A adrenergic agonist, in in vitro and in vivo models of urethral function." Life Sciences **70**(2): 181-197.

Obika, K., Shibata K., et al. (1995). "NS-49, a novel alpha 1a-adrenoceptor-selective agonist characterization using recombinant human alpha 1-adrenoceptors." European Journal of Pharmacology **291**(3): 327-334.

Obrink, A. & Bunne G. (1978). "The effect of alpha-adrenergic stimulation in stress incontinence." Scandinavian Journal Urology and Nephrology **12**(3): 205-208.

Ockenga, W. & Tikkanen R. (2015). "Revisiting the endocytosis of the m2 muscarinic acetylcholine receptor." Membranes **5**(2): 197-213.

Oehme, S., Mittag A., et al. (2015). "Agonist-induced ß2-adrenoceptor desensitization and downregulation enhance pro-inflammatory cytokine release in human bronchial epithelial cells." Pulmonary Pharmacology and Therapeutics **30**: 110-120.

Oh, S.J., Lee K.H., et al. (2000). "Active properties of the urinary bladder: in vitro comparative studies between adult and neonatal rats." British Journal of Urology **85**(9): 1126-1133.

Ohlstein, E.H., von Keitz A. & Michel M.C. (2012). "A multicenter, double-blind, randomized, placebo-controlled trial of the ß3-adrenoceptor agonist solabegron for overactive bladder." European Urology **62**(5): 834-840.

Opazo A., Lecea B. et al., (2009). "A comparative study of structure and function of the longitudinal muscle of the anal canal and the internal anal sphincter in pigs." Diseases of the colon and Rectum **52**(11):1902-1911.

Opazo, A., Lecea B., et al. (2011). "Specific and complementary roles for nitric oxide and ATP in the inhibitory motor pathways to rat internal anal sphincter." Neurogastroenterology and Motility **23**(1): e11-e25.

Orstavik, S., Natarajan V., et al. (1997). "Characterization of the human gene encoding the type I alpha and type I beta cGMP-dependent protein kinase (PRKG1)." Genomics **42**(2): 311-318.

Oshita, M., Hiraoka Y. & Watanabe Y. (1997). "Characterization of beta-adrenoceptors in urinary bladder: comparison between rat and rabbit." British Journal of Pharmacology **122**(8): 1720-1724.

Otsuka, A., Shinbo H., et al. (2008). "Expression and functional role of beta-adrenoceptors in the human urinary bladder urothelium." Naunyn Schmiedeberg's Archives of Pharmacology **377**: 473–481.

Owaki, H., Sadahiro S. & Takaki M. (2015). "Characterizations of the a1-adrenoceptor subtypes mediating contractions of the human internal anal sphincter." Journal of Pharmacological sciences **127**(4): 424-429.

Ozaki, H., Stevens R.J., et al. (1991). "Simultaneous measurement of membrane potential, cytosolic Ca2+, and tension in intact smooth muscles." The American Journal of Physiology **260**(5 Pt 1): C917-C925.

Ozawa, H., Chancellor M.B. et al. (1999). "Effect of intravesical nitric oxide therapy on cyclophosphamide-induced cystitis." The Journal of Urology **162**(6): 2211–2216.

Padykula, H.A. & Gauthier G.F. (1970). "The ultrastructure of the neuromuscular junctions of mammalian red, white, and intermediate skeletal muscle fibers." Journal of Cell Biology **46**(1):21-41.

Pager, C.K., Solomon M.J., et al. (2002). Long-term outcomes of pelvic floor exercise and biofeedback treatment for patients with fecal incontinence. Diseases of the Colon and Rectum **45**(8): 997-1003.

Palea, S., Toson G., et al. (1998). "Pharmacological characterization of thromboxane and prostanoid receptors in human isolated urinary bladder." British Journal of Pharmacology **124**(5): 865-872.

Palmer, K.R., Corbett C.L. & Holdsworth C.D. (1980). "Double-blind cross-over study comparing loperamide, codeine and diphenoxylate in the treatment of chronic diarrhea." Gastroenterology **79**(6): 1272-1275.

Palmqvist, M., Ibsen T., et al. (1999). “Comparison of the relative efficacy of formoterol and salmeterol in asthmatic patients.” American Journal of Respiratory and Critical Care Medicine **160**(1): 244-249.

Pandit, M., DeLancey J.O., et al. (2000). “Quantification of intramuscular nerves within the female striated urogenital sphincter muscle.” Obstetrics and Gynecology **95**(6 pt 1): 797–800.

Pandita, R.K. & Andersson K.E. (2002). “Intravesical adenosine triphosphate stimulates the micturition reflex in awake, freely moving rats.” Journal of Urology **168**(3): 1230-1234.

Pandita, R.K, Mizusawa H., & Andersson K. (2000). “Intravesical Oxyhemoglobin initiates bladder overactivity in conscious, normal rats.” Journal of Urology **164**(2): 545–550.

Paner, G.P., Ro J.Y. et al. (2007). “Further characterization of the muscle layers and lamina propria of the urinary bladder by systematic histologic mapping: implications for pathologic staging of invasive urothelial carcinoma.” American Journal of Surgical Pathology **31**(9): 1420-1429.

Paner, G.P., Shen S.S. et al. (2009). “Diagnostic utility of antibody to smoothelin in the distinction of muscularis propria from muscularis mucosae of the urinary bladder: a potential ancillary tool in the pathologic staging of invasive urothelial carcinoma.” American Journal of Surgical Pathology **33**(1): 91-98.

Pankratov, Y. & Lalo U. (2015). “Role for astroglial a1-adrenoreceptors in gliotransmission and control of synaptic plasticity in the neocortex.” Frontiers in Cellular Neuroscience **9**: 230.

Parajuli, S.P., Provence A. & Petkov G.V. (2014). “Prostaglandin E2 excitatory effects on guinea pig urinary bladder smooth muscle: a novel regulatory mechanism mediated by large-conductance voltage- and Ca2+-activated K+ channels.” European Journal of Pharmacology **738**: 179-185.

Park, K.S., Cha SK., et al. (2006). “An alpha3beta4 subunit combination acts as a major functional nicotinic acetylcholine receptor in male rat pelvic ganglion neurons.” Pfluger's Archive **452**(6): 775-783.

Park, J.S., Kang S.B., et al. (2015). “The efficacy and adverse effects of topical phenylephrine for anal incontinence after low anterior resection in patients with rectal cancer.” International Journal of Colorectal Diseases **22**(11): 1319–1324.

Park, K., Park S. & Jackson M.W. (2013). “The inhibitory effects of antimuscarinic autoantibodies in the sera of primary Sjogren syndrome patients on the gastrointestinal motility.” Molecular Immunology **56**(4): 583-587.

Parks, A. G., Fishlock D. J., et al. (1969). “Preiminary investigation of the pharmacology of the human internal anal sphincter.” Gut **10**(8): 674-677.

Parsons, C.L., Greenberger M., et al. (1998). “The role of urinary potassium in the pathogenesis and diagnosis of interstitial cystitis.” Journal of Urology **159**(6): 1862-1866.

Patel, C.A. & Rattan S. (2006). "Spontaneously tonic smooth muscle has characteristically higher levels of RhoA/ROK compared with the phasic smooth muscle." American Journal of Physiology; Gastrointestinal and Liver Physiology 291(5): G830-G837.

Patel, C.A. & Rattan S. (2007). "Cellular regulation of basal tone in internal anal sphincter smooth muscle by RhoA/ROCK." American Journal of Physiology; Gastrointestinal and Liver Physiology 292(6): G1747-G1756.

Patel, A.K. & Chapple C. R. (2008). "Anatomy of the lower urinary tract." Surgery **26**(4): 127-132.

Patriarchi, T., Qian H., et al. (2016). "Phosphorylation of Cav1.2 on S1928 uncouples the L-type Ca2+ channel from the ß2 adrenergic receptor." EMBO Journal **e201593409**.

Pediani, J.D., Colston J.F., et al. (2005). "Beta-arrestin-dependent spontaneous alpha1a-adrenoceptor endocytosis causes intracellular transportation of alpha-blockers via recycling compartments." Molecular Pharmacology **67**(4): 992-1004.

Pel, J.J., van Asselt E. & van Mastrigt R. (2006). "Contractile properties of the proximal urethra and bladder in female pig: morphology and function." Neurourology and Urodynamics **25**(1): 70-77.

Peng, F., Wu D., et al. (2008). "RhoA/Rho-kinase contribute to the pathogenesis of diabetic renal disease." Diabetes **57**(6): 1683-1692.

Penn, R. B., Panettieri R. A., & Benovic, J. L. (1998). "Mechanisms of Acute Desensitization of the β2AR–Adenylyl Cyclase Pathway in Human Airway Smooth Muscle." American Journal of Respiratory Cell and Molecular Biology **19**(2): 338-348.

Penninckx, F., Kerremans R., & Beckers, J. (1973). "Pharmacological characteristics of the non-striated anorectal musculature in cats." Gut **14**(5): 393-398.

Pera, T. & Penn R., B. (2014). "Crosstalk between beta-2-adrenoceptor and muscarinic acetylcholine receptors in the airway." Current Opinion in Pharmacology **16**: 72-81.

Perez, D. M., Piascik, M. T., & Graham, R. M. (1991). Solution-phase library screening for the identification of rare clones: isolation of an a1D-adrenergic receptor cDNA. Molecular Pharmacology **40**: 876- 883.

Perez-Aso, M., Segura V., et al. (2013). "The three a1-adrenoceptor subtypes show different spatio-temporal mechanisms of internalization and ERK1/2 phosphorylation." Biochimica et biophysica acta **1833**(10): 2322-2333.

Persson, K., Alm P., et al. (1993). "Nitric oxide synthase in pig lower urinary tract: immunohistochemistry, NADPH diaphorase histochemistry and functional effects." British Journal of Pharmacology **110**(2): 521-530.

Perucchini, D., DeLancey J. O. L., et al. (2002a). "Age effects on urethral striated muscle I. changes in number and diameter of striated muscle fibers in the ventral urethra." American Journal of Obstetrics and Gynecology **186**(3): 351-355.

Perucchini, D., DeLancey J.O., et al. (2002b). "Age effects on urethral striated muscle. II. Anatomic location of muscle loss." American Journal of Obstetrics and Gynecology **186**: 356–360.

Petkov, G.V. & Nelson M.T. (2005). "Differential regulation of Ca2+-activated K+ channels by beta-adrenoceptors in guinea pig urinary bladder smooth muscle." American Journal of Physiology; Cell Physiology **288** (6): C1255-1263.

Pfisterer, M.H., Griffiths D.J., et al. (2006). "The effect of age on lower urinary tract function: a study in women." Journal of American Geriatrics Society **54**(3): 405-412.

Pidsudko, Z. (2004). "Distribution and chemical coding of neurons in intramural ganglia of the porcine urinary bladder trigone." Folia Histochemica et Cytobiologica **42**(1): 3-11.

Pierce, K. L., Premont R. T., & Lefkowitz R. J. (2002). "Seven-transmembrane receptors." Nature Reviews of Molecular Cell Biology **3**(9): 639-650.

Pinedo, G., Garcia E., et al. (2009). "Are topical oestrogens useful in faecal incontinence? Double-blind randomized trial." Colorectal Diseases **11**(4): 390–393.

Pinedo, G., Zarate A.J., et al. (2012). "New treatment for faecal incontinence using zinc-aluminium ointment: a double-blind randomized trial." Colorectal Disease **14**(5): 596-598.

Ping, P., Anzai T., et al. (1997). "Adenylyl cyclase and G protein receptor kinase expression during development of heart failure." American Journal of Physiology **273**(2 Pt 2): H707-H717.

Pinna, C., Puglisi L., & Burnstock G. (1998). "ATP and vasoactive intestinal polypeptide relaxant responses in hamster isolated proximal urethra." British Journal of Pharmacology **124**(6): 1069-1074.

Pinna, C., Eberini I., et al. (1999). "Presence of constitutive endothelial nitric oxide synthase immunoreactivity in urothelial cells of hamster proximal urethra." European Journal of Pharmacology **367**(1): 85-89.

Pinna, C., Ventura S., et al. (1996). "A pharmacological and histochemical study of hamster urethra and the role of urothelium." British Journal of Pharmacology **119**(4): 655-662.

Piotrowska, A.P., Solari V. & Puri P. (2003). "Distribution of interstitial cells of Cajal in the internal anal sphincter of patients with internal anal sphincter achalasia and Hirschsprung disease." Archives of Pathology and Laboratory Medicine **127**(9): 1192-1195.

Pippig, S., Andexinger S. & Lohse M.J. (1995). “Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitization.” Molecular Pharmacology **47**(4): 666-676.

Pitcher, J.A., Touhara K., et al. (1995). “Pleckstrin homology domain-mediated membrane association and activation of the beta-adrenergic receptor kinase requires coordinate interaction with G beta gamma subunits and lipid.” Journal of Biological Chemistry **270**(20): 11707-11710.

Pitt, J., Craggs M.M., et al. (2000). "Alpha-1 adrenoceptor blockade: potential new treatment for anal fissures." Diseases of the Colon and Rectum **43**(6):800-803.

Pitt, J., Dawson P.M., et al. (2001). "A double-blind randomized placebo-controlled trial of oral indoramin to treat chronic anal fissure." Colorectal Disease **3**(3):165-168.

Pittman, R.N., Rabin R.A. et al. (1984). "Desensitization of beta-adrenergic receptor-coupled adenylate cyclase activity. Differences following exposure of cells to two full agonists." Biochemical Pharmacology **33**(22):3579-3584.

Poh, A., Tan K.Y. & Seow-Choen F. (2010). “Innovations in chronic anal fissure treatment: A systematic review.” World Journal of Gastrointestinal Surgery **2**(7): 231-241.

Poley, R.N., Dosier C.R., et al. (2008). “Stimulated calcium entry and constitutive RhoA kinase activity cause stretch-induced detrusor contraction.” European Journal of Pharmacology **599**(1-3): 137-145.

Ponglowhapan, S., Church D.B. & Khalid M. (2009). "Expression of cyclooxygenase-2 in the canine lower urinary tract with regard to the effects of gonadal status and gender." Theriogenology **71**(8):1276-128.

Ponglowhapan, S., Church D. B., & Khalid M. (2010). “Expression of prostaglandin E2 receptor subtypes in the canine lower urinary tract varies according to the gonadal status and gender.” Theriogenology **74**(8): 1450-1466.

Pouokam, E. & Diener M. (2011). “Mechanisms of actions of hydrogen sulphide on rat distal colonic epithelium.” British Journal of Pharmacology **162**(2): 392-404.

Pradidarcheep, W., Wallner C., et al. (2011). “Anatomy and histology of the lower urinary tract.” Handbook of Experimental Pharmacology **202**: 117–148.

Premont, R. T., & Gainetdinov R. R. (2007). “Physiological roles of G protein-coupled receptor kinases and arrestins.” Annual Review of Physiology **69**: (pp. 511-534).

Price, R. R., Morris D. P., et al. (2002). “Acute agonist-mediated desensitization of the human alpha 1a-adrenergic receptor is primarily independent of carboxyl terminus regulation: implications for regulation of alpha 1aAR splice variants.” Journal of Biological Chemistry **277**(11): 9570-9579.

Pronin, A. N., & Benovic J. L. (1997). “Regulation of the G protein-coupled receptor kinase GRK5 by protein kinase C.” Journal of Biological Chemistry **272**(6): 3806-3812.

Pronin, A.N., Satpaev D.K., et al. (1997). “Regulation of G protein-coupled receptor kinases by calmodulin and localization of the calmodulin binding domain.” Journal of Biological Chemistry **272**(29): 18273-18280.

Pronin A.N., Carman C.V. & Benovic J.L. (1998). “Structure-function analysis of G protein-coupled receptor kinase-5. Role of the carboxyl terminus in kinase regulation.” Journal of Biological Chemistry 273(47): 31510-31518.

Propping, S., Wuest M., et al. (2013). “Mucosa of human detrusor impairs contraction and ß-adrenoceptor-mediated relaxation.” British Journal of Urology International **112**(8): 1215-1222.

Propping, S., Roedel M., et al. (2015a). “Pharmacological Modulation of Mucosa-Related Impairment of ß-Adrenoceptor-Mediated Relaxation in Human Detrusor.” Urology International **95**(3): 300-308.

Propping, S., Newe M., et al. (2015b). “Mucosa of murine detrusor impairs ß2 -adrenoceptor-mediated relaxation.” Neurourology and Urodynamics **34**(6): 592-597.

Puetz, S., Lubomirov L. T., & Pfitzer, G. (2009). “Regulation of smooth muscle contraction by small GTPases.” Physiology **24**: 342-356.

Putney, J.W. Jr. (1986). “A model for receptor-regulated calcium entry.” Cell Calcium **7**(1): 1-12.

Putney, J.W. Jr. & McKay R.R. (1999). “Capacitative calcium entry channels.” Bioassays **21**(1): 38-46.

Qu, Z.D., Thacker M., et al. (2008). “Immunohistochemical analysis of neuron types in the mouse small intestine.” Cell and Tissue Research **334**(2): 147-161.

Racchi, H., Irarrázabal M.J. et al. (1999). "Adenosine 5'-triphosphate and neuropeptide Y are co-transmitters in conjunction with noradrenaline in the human saphenous vein." British Journal of Pharmacology **126**(5):1175-1855.

Radley, S.C., Chapple C.R., et al. (2001). “Effect of methoxamine on maximum urethral pressure in women with genuine stress incontinence: a placebo-controlled, double-blind crossover study.” Neurourology and urodynamics **20**(1): 43-52.

Radziszewski, P., Ekblad E., et al. (1996). “Distribution of neuropeptide-, tyrosine hydroxylase- and nitric oxide synthase containing nerve fibers in the external urethral sphincter of the rat.” Scandinavian Journal of Urology and Nephrology **179**: 81-85.

Rae, M.G. & Muir T.C. (1996). "Neuronal mediators of inhibitory junction potentials and relaxation in the guinea-pig internal anal sphincter." The journal of Physiology **493** (Pt 2): 517-527.

Raghavan, S., Miyasaka E.A., et al. (2010). "Successful implantation of physiologically functional bioengineered mouse internal anal sphincter." American Journal of Physiology; Gastrointestinal and liver physiology **299**(2): G430-G439.

Raghavan, S., Gilmont R.R., et al. (2011). "Successful implantation of bioengineered, intrinsically innervated, human internal anal sphincter." Gastroeneterology **141**(1): 310-319.

Raghavan, S., Miyasaka E.A., et al. (2014). "Perianal implantation of bioengineered human internal anal sphincter constructs intrinsically innervated with human neural progenitor cells." Surgery **155**(4): 668-674.

Ragionieri, L., Ravanetti F., et al. (2016). "Morphological analysis of the urethral muscle of the male pig with relevance to urinary continence and micturition." Journal of Anatomy **228**(3): 511-519.

Rahnama'I, M.S., van Koeveringe G.A., et al. (2010). "Prostaglandin receptor EP1 and EP2 site in guinea pig bladder urothelium and lamina propria." Journal of Urology **183**(3): 1241-1247.

Ram, E., Vishne T., et al. (2007). Anal dilatation versus left lateral sphincterotomy for chronic anal fissure: a prospective randomized study. Techniques in Coloproctology [Epub ahead of print].

Ramachandran, R., Noorbakhsh F., et al. (2012). "Targeting proteinase-activated receptors: therapeutic potential and challenges." Nature Reviews Drug Discovery **11**(1): 69-86.

Ramalingam, T., Durlu-Kandilci N.T. & Brading A.F. (2010). "A comparison of the contractile properties of smooth muscle from pig urethra and internal anal sphincter." Neurourology and Urodynamics **29**(7): 1326-1331.

Ramsay D., Carr I.C., et al. (2004). "High-affinity interactions between human alpha1A-adrenoceptor C-terminal splice variants produce homo- and heterodimers but do not generate the alpha1L-adrenoceptor." Molecular pharmacology **66**(2): 228-239.

Ranson, R.N. & Saffrey M.J. (2015). "Neurogenic mechanisms in bladder and bowel ageing." Biogerontology **16**(2): 265-284.

Rao, S.S. & American College of Gastroenterology Practice Parameters Committee. (2004). "Diagnosis and management of fecal incontinence. American College of Gastroenterology Practice Parameters Committee." American Journal of Gastroenetrology **99**(8): 1585-1604.

Rathi, S., Kazerounian S., et al. (2003). "Functional and molecular characterization of beta-adrenoceptors in the internal anal sphincter." Journal of Pharmacology and Experimental Therapeutics **305**(2): 615-624.

Rattan, S. (2005). "The internal anal sphincter: regulation of smooth muscle tone and relaxation." Neurogastroenterology and Motility **17** Suppl 1: 50-59.

Rattan, S. & Chakder S. (1992). "Role of nitric oxide as a mediator of internal anal sphincter relaxation." American Journal of Physiology **262**(1 Pt 1): G107-G112.

Rattan, S. & Chakder S. (1993). "Inhibitory effect of CO on internal anal sphincter: heme oxygenase inhibitor inhibits NANC relaxation." American Journal of Physiology **265**(4 Pt 1): G799-G804.

Rattan, S. & Chakder S. (2000). "Influence of heme oxygenase inhibitors on the basal tissue enzymatic activity and smooth muscle relaxation of internal anal sphincter." Journal of Pharmacology and Experimental Therapeutics **294**(3): 1009-1016.

Rattan, S., & Singh, J. (2011). "Basal internal anal sphincter tone, inhibitory neurotransmission, and other factors contributing to the maintenance of high pressures in the anal canal." Neurogastroenterology and Motility **23**(1): 3-7.

Rattan, S. & Singh J. (2012). "RhoA/ROCK pathway is the major molecular determinant of basal tone in intact human internal anal sphincter." American Journal of Physiology; Gastrointestinal and Liver Physiology **302**(7): G664-G675.

Rattan, S., Sarkar A. & Chakder S. (1992). "Nitric oxide pathway in rectoanal inhibitory reflex of opossum internal anal sphincter." Gastroeneterology **103**(1): 43-50.

Rattan, S., Al Haj R. & De Godoy M.A. (2004). "Mechanism of internal anal sphincter relaxation by CORM-1, authentic CO, and NANC nerve stimulation." American Journal of Physiology; Gastrointestinal and Liver Physiology **287**(3): G605-G611.

Rattan, S., De Godoy M.A. & Patel C.A. (2006). "Rho kinase as a novel molecular therapeutic target for hypertensive internal anal sphincter." Gastroenetrology **131**(1): 108-116.

Rattan, S., Regan R.F., et al. (2005). "Nitric oxide not carbon monoxide mediates nonadrenergic noncholinergic relaxation in the murine internal anal sphincter." Gastroenetrology **129**(6): 1954-1966.

Rattan, S., Singh J., et al. (2015). "Nature of extracellular signal that triggers RhoA/ROCK activation for the basal internal anal sphincter tone in humans." American Journal of Physiology; Gastrointestinal and Liver Physiology **308**(11): G924-G933.

Ratz, P.H & Miner A.S. (2009). "Role of protein kinase Czeta and calcium entry in KCl-induced vascular smooth muscle calcium sensitization and feedback control of cellular calcium levels." The Journal of Pharamcology and Experimental Therapeutics **328**(2): 399-408.

Ratz, P. H., Miner A. S., & Barbour S. E. (2009). Calcium-independent phospholipase A2 participates in KCl-induced calcium sensitization of vascular smooth muscle. Cell Calcium **46**(1), 65-72.

Rayment, S.J., Eames T., et al. (2010). “Investigation of the distribution and function of alpha-adrenoceptors in the sheep isolated internal anal sphincter.” British Journal of Pharmacology **160**(7): 1727-1740.

Rayment, S.J., Simpson J.A., et al. (2014). “Dual effects of a2 -adrenoceptors in modulating myogenic tone in sheep isolated internal anal sphincter.” Neurogastroenetrology and Motility **26**(8): 1095-1103.

Rayner, V. (1979). “Characteristics of the internal anal sphincter and the rectum of the vervet monkey.” Journal of Physiology **286**: 383-399.

Redaelli, M., Ricatti M.J., et al. (2015). “Serotonin and noradrenaline reuptake inhibitors improve micturition control in mice.” Plose One **10**(3): e0121883.

Remes-Troche, J.M., De-Ocampo S., et al. (2010). “Rectoanal reflexes and sensorimotor response in rectal hyposensitivity.” Diseases of the Colon and Rectum **57**(7): 1047-1054.

Rengo, G., Perrone-Filardi P., et al. (2012a). “Targeting the ß-adrenergic receptor system through G-protein coupled receptor kinase 2: a new paradigm from therapy and prognostic evaluation in heart failure: from bench to bedside.” Circulation, Heart Failure **5**(3): 385-391.

Rengo, G., Lymperopoulos A., et al. (2012b). “Blockade of ß-adrenoceptors restores the GRK2-mediated adrenal a2-adrenoceptor-catecholamine production axis in heart failure.” British Journal of Pharmacology **166**(8): 2430-2340.

Rengo, G., Zincarelli C., et al. (2012c). “Myocardial ß2-adrenoceptor gene delivery promotes coordinated cardiac adaptive remodelling and angiogenesis in heart failure.” British Journal of Pharmacology **166**(8): 2348-2361.

Rengo, G., Galasso G., et al. (2014). “Reduction of lymphocyte G protein-coupled receptor kinase-2 (GRK2) after exercise training predicts survival in patients with heart failure.” European Journal of Preventive Cardiology **21**(1): 4-11.

Rich, A., Farrugia G. & Rae J.L. (1994). "Carbon monoxide stimulates a potassium-selective current in rabbit corneal epithelial cells." American Journal of Physiology **267**(2 pt 1):C435-C442.

Robain, G., Combrisson H. & Mazieres L. (2001). “Bladder response to urethral flow in the awake ewe.” Neurourology and Urodynamics **20**(5): 641–649.

Robinson, J. D., & Pitcher J. A. (2013). “G protein-coupled receptor kinase 2 (GRK2) is a Rho-activated scaffold protein for the ERK MAP kinase cascade.” Cell Signal **25**(12): 2831-2839.

Rodríguez-Pérez, C. E., Calvo-Ochoa E., et al. (2009). “Receptor tyrosine kinases regulate α1D-adrenoceptor signaling properties: Phosphorylation and desensitization.” The International Journal of Biochemistry & Cell Biology **41**(6): 1276-1283.

Rogers, J. (1992). "Testing for and the role of anal and rectal sensation." Bailliere's Clinical Gastroenterology **6**(1): 179-91.

Rohde, S., Sabri A., et al. (2000). "The alpha(1)-adrenoceptor subtype- and protein kinase C isoform-dependence of Norepinephrine's actions in cardiomyocytes." Journal of Molecular and Cellular Cardiology **32**(7): 1193-1209.

Rojanathammanee, L., Harmon E.B., et al. (2009). "The 27-kDa heat shock protein confers cytoprotective effects through a beta 2-adrenergic receptor agonist initiated complex with beta-arrestin." Molecular Pharmacology **75**: 855–865.

Rokosh, D.G., Stewart A.F., et al. (1996). "Alpha1-adrenergic receptor subtype mRNAs are differentially regulated by alpha1-adrenergic and other hypertrophic stimuli in cardiac myocytes in culture and in vivo. Repression of alpha1B and alpha1D but induction of alpha1C." Journal of Biological Chemistry **271**(10): 5839-5843.

Romih, R., Korosec P., et al. (2005). "Differentiation of epithelial cells in the urinary tract. Cell and Tissues Research **320**(2): 259-268.

Roosen, A., Wu C., et al. (2008). "Synergistic effects in neuromuscular activation and calcium-sensitization in the bladder trigone." British Journal of Urology International **101**(5): 610-614.

Roosen, A., Wu C., et al. (2009). "Characteristics of spontaneous activity in the bladder trigone." European Urology **56**(2): 346-353.

Root, J.A., Davey D.A. & Af-Forselles K.J. (2015). "Prostanoid receptors mediating contraction in rat, macaque and human bladder smooth muscle in vitro." European Journal of Pharmacology **769**: 274-279.

Rosethorne, E.M., Bradley M.E., et al. (2015). "Functional desensitization of the ß 2 adrenoceptor is not dependent on agonist efficacy." Pharmacology research and perspectives **3**(1): e00101.

Ruan, Y., Kan H., et al. (1998). "Alpha-1A adrenergic receptor stimulation with phenylephrine promotes arachidonic acid release by activation of phospholipase D in rat-1 fibroblasts: inhibition by protein kinase A." Journal of Pharmacology and Experimental Therapeutics **284**(2): 576-585.

Rud, T. (1980). "Urethral pressure profile in continent women from childhood to old age." Acta Obstetricia et gynecological Scandinavia **59**(4): 331-335.

Rumzhum, N.N., Rahman M.M., et al. (2016). "Effect of Sphingosine 1-Phosphate on Cyclo-Oxygenase-2 Expression, Prostaglandin E2 Secretion, and ß2-Adrenergic Receptor Desensitization." American Journal of Respiratory Cell and Molecular Biology **54**(1):128-135.

Ruggieri, M.R Sr. & Braverman A.S. (2006). "Regulation of bladder muscarinic receptor subtypes by experimental pathologies." Autonomic and Autacoid Pharmacology **26**(3): 311-325.

Said, S.I. & Mutt V. (1970). "Polypeptide with broad biological activity: isolation from small intestine." Science **169**(3951): 1217-1218.

Sallese, M., Iacovelli L., et al. (2000). "Regulation of G protein-coupled receptor kinase subtypes by calcium sensor proteins." Biochimica et Biophysica Acta - Molecular Cell Research **1498**(2-3): 112-121.

Samoshkin, A., Convertino M., et al. (2015). "Structural and functional interactions between six-transmembrane μ-opioid receptors and ß2-adrenoreceptors modulate opioid signaling." Scientific Reports **5**: 18198.

Santoro, G.A., Infantino A., et al. (2012). Sacral nerve stimulation for fecal incontinence related to external sphincter atrophy. Diseases of the Colon and Rectum **55**(5) 797–805.

Sato, S., Shirato K., et al. (2011). "Synthesized glucocorticoid, dexamethasone regulates the expressions of ß2-adrenoceptor and glucocorticoid receptor mRNAs but not proteins in slow-twitch soleus muscle of rats." The Journal of Toxicological Sciences **36**(4): 479-486.

Sauzeau, V., Le Jeune H., et al. (2000). "Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca2+ sensitization of contraction in vascular smooth muscle." Journal of Biological Chemistry **275**(28): 21722-21729.

Save, S. & Persson K. (2010). "Extracellular ATP and P2Y receptor activation induce a proinflammatory host response in the human urinary tract." Infection and Immunology **78**(8): 3609-3615.

Schagen van Leeuwen, J.H., Lange R.R., et al. (2008). "Efficacy and safety of duloxetine in elderly women with stress urinary incontinence or stress-predominant mixed urinary incontinence." Maturitas **60**(2): 138-147.

Scharli, A.F. & Kiesewetter W.B. (1970). Defecation and continence: some new concepts. Diseases of the Colon and Rectum **13**(2): 81-107.

Schicho, R, Krueger D. et al. (2006). "Hydrogen sulfide is a novel prosecretory neuromodulator in the Guinea-pig and human colon." Gastroenterology **131**(5):1542-1552.

Schneider, T., Hein P. & Michel M.C. (2004a). "Signal transduction underlying carbachol-induced contraction of rat urinary bladder. I. Phospholipases and Ca2+ sources." Journal of Pharmacology and Experimental Therapeutics **308**: 47–53.

Schneider, T., Fetscher C., et al. (2004b). "Signal transduction underlying carbachol-induced contraction of human urinary bladder." Journal of Pharmacology and Experimental Therapeutics **309**(3): 1148-1153.

Scholefield, J.H., Bock J.U., et al. (2003). "A dose finding study with 0.1%, 0.2%, and 0.4% glyceryl trinitrate ointment in patients with chronic anal fissures." Gut **52**(2):264-269.

Schröder, A., Newgreen D. & Andersson K.E. (2004). "Detrusor responses to prostaglandin E2 and bladder outlet obstruction in wild-type and Ep1 receptor knockout mice." Journal of Urology **172**(3): 1166-1170.

Schwinn, D., Lomasney, J., et al. (1990). Molecular cloning and expression of the cDNA for a novel a-adrenergic receptor subtype. Journal of Biological Chemitry **265**:8183 - 8189.

Schwinn, D. A., Johnson, G. I., et al. (1995). Cloning and pharmacological characterization of human alpha1 adrenergic receptors: sequence corrections and direct comparison with other species homologues. Journal of Pharmacology and Experimental Therapeutics **272**: 134-142.

Scola, A.M., Chong L.K., et al. (2004). "Desensitisation of mast cell beta2-adrenoceptor-mediated responses by salmeterol and formoterol." British Journal of Pharmacology **141**(1): 163-171.

Seachrist, J.L., Anborgh P.H. & Ferguson S.S. (2000). "Beta 2-adrenergic receptor internalization, endosomal sorting, and plasma membrane recycling are regulated by rab GTPases." Journal of Biological Chemistry **275**(35): 27221-27228.

Sears, M.R., Taylor D.R., et al. (1990). "Regular inhaled beta-agonist treatment in bronchial asthma." Lancet **336**(8728): 1391-1396.

Segev, G., Westropp J.L., et al. (2015). "Changes in blood pressure following escalating doses of phenylpropanolamine and a suggested protocol for monitoring." The Canadian Veterinary Journal **56**(1): 39-43.

Segura, V., Pérez-Aso M., et al. (2013). "Differences in the signaling pathways of a(1A)- and a(1B)-adrenoceptors are related to different endosomal targeting." Plos One **8**(5): e64996.

Seibold, A., Williams B., et al. (2000). "Localization of the sites mediating desensitization of the beta(2)-adrenergic receptor by the GRK pathway." Molecular Pharmacology **58**(5): 1162-1173.

Sellers, D.J., Yamanishi T., et al. (2000). "M3 muscarinic receptors but not M2 mediate contraction of the porcine detrusor muscle in vitro." Journal of Autonomic Pharmacology **20**(3): 171-176.

Seok, Y.M., Baek I., et al. (2008). "Isoflavone attenuates vascular contraction through inhibition of the RhoA/Rho-kinase signaling pathway." Journal of Pharmacology Experimental Therapeutics **326**: 991–998.

Sergeant, G.P., Hollywood M.A., et al. (2000). "Specialised pacemaking cells in the rabbit urethra." Journal of Physiology **526**(2): 359-366.

Shabir, S., Cross W., et al. (2013). "Functional expression of purinergic P2 receptors and transient receptor potential channels by the human urothelium." American Journal of Physiology; Renal physiology **305**(3): F396-F406.

Shafei, M., Thornbury K.D., et al. (2003). "Relative contributions of calcium influx and calcium stores to myogenic tone in the rat urethra." Journal of Physiology **548P**: O15.

Shafik, A. (1999). "A study of the continence mechanism of the external urethral sphincter with identification of the voluntary urinary inhibition reflex." Journal of Urology **162**(6): 1967-1971.

Shafik, A., Shafik A.A., et al. (2003). "Role of positive urethrovesical feedback in vesical evacuation. The concept of a second micturition reflex: the urethrovesical reflex." World Journal of Urology **21**: 167–170.

Shafik, A., El Sibai O. & Ahmed I. (2006). "The identification of specialized pacemaking cells in the anal sphincters." International Journal of Colorectal Disease **21**(5): 453-457.

Shah, A.P., Mevcha A., et al. (2014). "Continence and micturition: an anatomical basis." Clinical Anatomy **27**(8):1275-1283.

Shah, B. J., Chokhavatia S., & Rose, S. (2012). "Fecal incontinence in the elderly: FAQ." American Journal of Gastroenterology **107**(11): 1635-1646.

Shahab, N., Kajioka S., et al. (2012a). "Functional role of muscarinic receptor subtypes in calcium sensitization and their contribution to rho-kinase and protein kinase C pathways in contraction of human detrusor smooth muscle." Urology **79**(5): 1184.e7-e13.

Shahab, N., Kajioka S., et al. (2012b). "Obstruction enhances rho-kinase pathway and diminishes protein kinase C pathway in carbachol-induced calcium sensitization in contraction of a-toxin permeabilized guinea pig detrusor smooth muscle." Neurourology and Urodynamics **31**(4): 593-599.

Shapiro, E., Tsitlijk J.E. & Lepor H. (1987). "α_2 adrenergic receptors in canine prostate: biochemical and functional correlations." Journal of Urology **137**: 565–570.

Shibata, K., Hirasawa A., et al. (1996). "Alpha 1a-adrenoceptor polymorphism: pharmacological characterization and association with benign prostatic hypertrophy." British Journal of Pharmacology **118**(6): 1403-1408.

Shin, C.W., Kim S.D. & Cho W.Y. (2009). "The prevalence and management of urinary incontinence in elderly patients at sanatorium in Busan area." Korean Journal of Urology **50**: 450–456.

Shirazi, A., Iizuka K., et al. (1994). "Purification and characterization of the mammalian myosin light chain phosphatase holoenzyme. The differential effects of the holoenzyme and its subunits on smooth muscle." Journal of Biological Chemistry **269**(50): 31598-31606.

Shum, W.W., Le G.Y., et al. (2003). Involvement of Rho-kinase in contraction of guinea-pig aorta induced by prostanoid EP3 receptor agonists. British Journal of pharmacology **139**(8):1449-1461.

Shuttleworth, C.W., Xue C., et al. (1993). "Immunohistochemical localization of 3',5'-cyclic guanosine monophosphate in the canine proximal colon: responses to nitric oxide and electrical stimulation of enteric inhibitory neurons." Neuroscience **56**(2): 513-522.

Sibley, D. R., Strasser R. H., et al. (1986). "Phosphorylation/dephosphorylation of the beta-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution." Proceedings of National Academy of Science USA **83**(24): 9408-9412.

Sienkiewicz, W., Kaleczyc J., et al. (2004). "Adrenergic, nitrergic and peptidergic innervation of the urethral muscle in the boar." Folia Histochemica et Cytobiologica **42**(2): 89-94.

Sigala, S., Mirabella G., et al. (2002). "Differential gene expression of cholinergic muscarinic receptor subtypes in male and female normal human urinary bladder." Urology **60**(4): 719-725.

Simpson, J.A., Bush D., et al. (2014). "A randomised, controlled, crossover study to investigate the safety and response of 1R,2S-methoxamine hydrochloride (NRL001) on anal function in healthy volunteers." Colorectal Diseases **16** Suppl 1: 5-15.

Sims, S.M., Chrones T. & Preiksaitis H.G. (2008). "Calcium sensitization in human esophageal muscle: role for RhoA kinase in maintenance of lower esophageal sphincter tone." Journal of Pharmacology and Experimental Therapeutics **327**(1): 178-186.

Singh, J., Kumar S. & Rattan S. (2015). "Bimodal effect of oxidative stress in internal anal sphincter smooth muscle." American Journal of Physiology; Gastrointestinal and Liver Physiology **309**(5): G292-G300.

Singh, J., Mehendiratta V., et al. (2009). "Immunoglobulins from scleroderma patients inhibit the muscarinic receptor activation in internal anal sphincter smooth muscle cells." American Journal of Physiology; Gastrointestinal and Liver Physiology **297**(6): G1206-G1213.

Singh, J., Kumar S., et al. (2014). "Aging-associated oxidative stress leads to decrease in IAS tone via RhoA/ROCK downregulation." American Journal of Physiology; Gastrointestinal and Liver Physiology **306**(11): G983-G991.

Siproudhis, L, Morcet J. & Laine F. (2007). "Elastomer implants in faecal incontinence: a blind, randomized placebo-controlled study." Alimentary Pharmacology and Therapeutics **25**(9): 1125-1132.

Siproudhis, L., Jones D., et al. (2014). "Libertas: rationale and study design of a multicentre, Phase II, double-blind, randomised, placebo-controlled investigation to evaluate the efficacy, safety and tolerability of locally applied NRL001 in patients with faecal incontinence." Colorectal Diseases **16** (Suppl 1): 59-66.

Sjödahl, J., Walter S.A., et al. (2015). “Combination therapy with biofeedback, loperamide, and stool-bulking agents is effective for the treatment of fecal incontinence in women - a randomized controlled trial.” Scandinavian Journal of Gastroenterology **50**(8): 965-974.

Smet, P.J., Jonavicius J., et al. (1996). “Distribution of nitric oxide synthase-immunoreactive nerves and identification of the cellular targets of nitric oxide in guinea-pig and human urinary bladder by cGMP immunohistochemistry.” Neuroscience **71**(2): 337-348.

Smith, E.G., Voyles W.F., et al. (2007). “Ageing and leg postjunctional alpha-adrenergic vasoconstrictor responsiveness in healthy men.” Journal of Physiology **582**(Pt 1): 63-71.

Smith, K. M., Macmillan J. B., & McGrath J. C. (1997). “Investigation of alpha1-adrenoceptor subtypes mediating vasoconstriction in rabbit cutaneous resistance arteries.” British Journal of Pharmacology **122**(5): 825-832.

Somara, S., Gilmont R.R., et al. (2009). “Bioengineered internal anal sphincter derived from isolated human internal anal sphincter smooth muscle cells.” Gastroeterology **137**(1): 53-61.

Somogyi, G.T., Tanowitz M., et al. (1996). “M1 muscarinic receptor-induced facilitation of ACh and noradrenaline release in the rat bladder is mediated by protein kinase C.” Journal of Physiology **496** (Pt 1): 245-254.

Sood R., Faubion S.S., et al. (2014). Prescribing menopausal hormone therapy: an evidence-based approach. International Journal of Women Health **6**:47-57.

Speakman, C.T., Hoyle C.H., et al. (1990). “Adrenergic control of the internal anal sphincter is abnormal in patients with idiopathic faecal incontinence.” British Journal of Surgery **77**(12): 1342-1344.

Speakman, C.T., Hoyle C.H., et al. (1992). “Decreased sensitivity of muscarinic but not 5-hydroxytryptamine receptors of the internal anal sphincter in neurogenic faecal incontinence.” British Journal of Surgery **79**(8): 829-832.

Speakman, C.T., Hoyle C.H., et al. (1993). “Abnormalities of innervation of internal anal sphincter in fecal incontinence.” Digestive Diseases and Sciences **38**(11): 1961-1969.

Sprossmann, F., Pankert P., et al. (2009). “Inducible knockout mutagenesis reveals compensatory mechanisms elicited by constitutive BK channel deficiency in overactive murine bladder.” FEBS Journal **276**(6): 1680-1697.

Stafford, R.E., Ashton-Miller J.A., et al. (2012). “Activation of the striated urethral sphincter to maintain continence during dynamic tasks in healthy men.” Neurourology and Urodynamics **31**(1): 36-43.

Stanasila, L., Perez J.B., et al. (2003). “Oligomerization of the alpha 1a- and alpha 1b-adrenergic receptor subtypes. Potential implications in receptor internalization.” Journal of Biological Chemistry **278**(41): 40239-40251.

Stanasila, L., Abuin L., et al. (2008). "Different internalization properties of the alpha1a- and alpha1b-adrenergic receptor subtypes: the potential role of receptor interaction with beta-arrestins and AP50." Molecular Pharmacology **74**(3): 562-573.

Steers, W.D. (2006). "Darifenacin: Pharmacology and clinical usage." Urological Clinics of North America **33**(4): 475-482.

Stoker, J. (2009). Anorectal and pelvic floor anatomy. Best Practice and Research; Clinical Gastroenterology **23**(4): 463-475.

Streng, T., Axelsson H.E., et al. (2008). "Distribution and function of the hydrogen sulfide-sensitive TRPA1 ion channel in rat urinary bladder." European Urology **53**(2): 391-399.

Streng, T., Santti R. & Andersson K.E. (2010). "Voiding effects mediated by a2-adrenoceptors in the anaesthetized male rat." British Journal of Urology **106**(10): 1546-1549.

Su, T.H., Morishima S., et al. (2008a). "Native profiles of alpha(1A)-adrenoceptor phenotypes in rabbit prostate." British Journal of Pharmacology **155**(6):906-912.

Su, X., Leon L.A., et al. (2008b). "Modulation of bladder function by prostaglandin EP3 receptors in the central nervous system." American Journal of Physiology; Renal Physiology **295**(4): F984-F994.

Sugaya, K., Roppolo J.R., et al. (1997). "The central neural pathways involved in micturition in the neonatal rat as revealed by the injection of pseudorabies virus into the urinary bladder." Neuroscience letters **223**(3): 197-200.

Sugaya, K., Ogawa Y. et al. (2003). "Ascending and descending brainstem neuronal activity during cystometry in decerebrate cats." Neurourology and Urodynamics **22**(4):343-350.

Sui, G.P., Wu C., & Fry C.H. (2001). "Inward calcium currents in cultured and freshly isolated detrusor muscle cells: evidence of a T-type calcium current." Journal of Urology **165**(2): 621-626.

Sui, G.P., Wu C. & Fry C.H. (2003). "A description of Ca2+ channels in human detrusor smooth muscle." British Journal of Urology International **92**(4): 476-482.

Sui, G.P., Wu C., et al. (2007). "The association between T-type Ca2+ current and outward current in isolated human detrusor cells from stable and overactive bladders." British Journal of Urology International **99**(2): 436-441.

Sui, G.P., Wu C., et al. (2008). "Modulation of bladder myofibroblast activity: implications for bladder function." American Journal of Physiology; renal Phyiology **295**(3): F688-F697.

Sui, G., Fry C.H., et al. (2009). "Aberrant Ca^{2+} oscillations in smooth muscle cells from overactive human bladders." Cell Calcium **45**(5): 456–564.

Sui, G., Fry C.H., et al. (2014). “Purinergic and muscarinic modulation of ATP release from the urothelium and its paracrine actions.” The American Journal of Physiology. Renal Physiology **306**(3): F286–F298.

Sun, W.M., Read N.W. & Miner PB. (1990). Relation between rectal sensation and anal function in normal subjects and patients with faecal incontinence. Gut **31**(9): 1056–1061.

Sun, Y. & Chai T.C. (2006). “Augmented extracellular ATP signaling in bladder urothelial cells from patients with interstitial cystitis.” American Journal of Physiology; Cell Physiology **290**(1): C27-C34.

Sung, V.W., Rogers M.L., et al. (2007). National trends and costs of surgical treatment for female fecal incontinence. American Journal of Obstetrics and Gynecology **197**(6):652.e1-e5.

Surks, H. K. (2007). “cGMP-dependent protein kinase I and smooth muscle relaxation: a tale of two isoforms.” Circulation Research **101**(11): 1078-1080.

Surks, H.K., Mochizuki N., et al. (1999). “Regulation of myosin phosphatase by a specific interaction with cGMP- dependent protein kinase Ialpha.” Science **286**(5444): 1583-1587.

Suzuki, F., Taniguchi T., et al. (2000). “Splice isoforms of alpha(1a)-adrenoceptor in rabbit.” British Journal of Pharmacology **129**(8): 1569-1576.

Suzuki, Y., Moriyama N., et al. (1999). “Age-related change of the role of α1L-adrenoceptor.” The Vascular System **33**(4): 347-354.

Sward, K., Dreja, K., et al. (2000). “Inhibition of Rho-associated kinase blocks agonist-induced Ca2+ sensitization of myosin phosphorylation and force in guinea-pig ileum." Journal of Physiology **522** (Pt 1): 33-49.

Taguchi, K., Yang M., et al. (1998). “Comparison of human alpha1-adrenoceptor subtype coupling to protein kinase C activation and related signalling pathways.” Naunyn-Schmiedeberg's Archives of Pharmacology **357**(2): 100-110.

Tai, C., Wang J. et al. (2009). "Brain switch for reflex micturition control detected by FMRI in rats." Journal of Neurophysiology **102**(5):2719-2730.

Takahashi, N., Shiomi H., et al. (2009). “Obstruction alters muscarinic receptor-coupled RhoA/Rho-kinase pathway in the urinary bladder of the rat.” Neurourology and Urodynamics **28**(3): 257-262.

Takasu, T., Ukai M., et al. (2007). “Effect of (R)-2-(2-aminothiazol-4-yl)-4'-{2-[(2-hydroxy-2-phenylethyl)amino]ethyl} acetanilide (YM178), a novel selective beta3-adrenoceptor agonist, on bladder function.” The Journal of Pharmacology and Experimental Therapeutics **321**(2): 642-647.

Takeda, M., Obara K., et al. (1999). "Evidence for beta3-adrenoceptor subtypes in relaxation of the human urinary bladder detrusor: analysis by molecular biological and pharmacological methods." Journal of Pharmacology and Experimental Therapeutics **288**(3): 1367-1373.

Takeda, H., Matsuzawa A., et al. (2003). "Functional characterization of beta-adrenoceptor subtypes in the canine and rat lower urinary tract." Journal of Urology **170**(2 Pt 1): 654-658.

Taki, N., Taniguchi T., et al. (1999). "Evidence for predominant mediation of α1-adrenoceptor in the tonus of entire urethra of women." Journal of Urology **162**(5): 1829-1832.

Takir, S., Ortaköylü G.Z., et al. (2015). "NaHS induces relaxation response in prostaglandin F(2a) precontracted bovine retinal arteries partially via K(v) and K(ir) channels." Experimental Eye Research **132**: 190-197.

Tanaka, E., Noguchi T., et al. (2012) Morphology of the epithelium of the lower rectum and the anal canal in the adult human. Medical Molecular Morphology **45**(2):72-79.

Tanaka, I., Nagase K., et al. (2010). "Improvement in neurogenic detrusor overactivity by peripheral C fiber's suppression with cyclooxygenase inhibitors." Journal of Urology **183**(2): 786-792.

Tanaka, Y., Koyama Y., et al. (2003). "Firing of micturition center neurons in the rat mesopontine tegmentum during urinary bladder contraction." Brain Research **965**(1-2):146-154.

Tanaka, Y., Horinouchi T., & Koike, K. (2005). "New insight into β-adrenoceptors in smooth muscle: distribution of receptor subtypes and molecular mechanisms triggering muscle relaxation." Clinical and Experimental Pharmacology and Physiology **32**(7): 503-514.

Tatsumiya, K., Yamanishi T., et al. (2009). "Effects of fasudil, a Rho-kinase inhibitor, on contraction of pig bladder tissues with or without urothelium." International Journal of Urology **16**(12): 959-966.

Teague, B., Asiedu S. & Moore P.K. (2002). "The smooth muscle relaxant effect of hydrogen sulphide in vitro: evidence for a physiological role to control intestinal contractility." British Journal of Pharmacology **137**(2): 139-145.

Teixeira, C. E., Jin L., et al. (2007). "Comparative pharmacological analysis of Rho-kinase inhibitors and identification of molecular components of Ca2+ sensitization in the rat lower urinary tract." Biochemical Pharmacology **74**(4): 647-658.

Tempest, H.V., Dixon A.K, et al. (2004). "P2X and P2X receptor expression in human bladder urothelium and changes in interstitial cystitis." British Journal of Urology International **93**(9): 1344-1348.

Templeman, L., Chapple C. R., & Chess-Williams, R. (2002). "Urothelium derived inhibitory factor and cross-talk among receptors in the trigone of the bladder of the pig." Journal of Urology **167**(2 Pt 1): 742-745.

Teng, J., Wang Z.Y. et al. (2008). "Roles of estrogen receptor alpha and beta in modulating urothelial cell proliferation." Endocrine-Related Cancer **15**(1): 351-364.

Tenhunen, R., Marver H.S., et al. (1969). "Microsomal heme oxygenase. Characterization of the enzyme." Journal of Biological Chemistry **244**(23): 6388-6394.

Testa, R., Guarneri L., et al. (1993). "Characterization of α1-adrenoceptor subtypes in prostate and prostatic urethra of rat, rabbit, dog and man." European Journal of Pharmacology **249**(3): 307-315.

Testa, R., Guarneri L., et al. (1997). Pharmacological characterization of the uroselective alpha-1 antagonist Rec 15/2739 (SB 216469): role of the alpha-1L adrenoceptor in tissue selectivity, part II. Journal of Pharmacology and Experimental Therapeutics **281**: 1284–1293.

Tetzschner, T., Sørensen M., et al. (1995). Pudendal nerve damage increases the risk of fecal incontinence in women with anal sphincter rupture after childbirth. Acta Obstetricia et Gynecologica Scandinavica **74**(6) 434–440.

Thangaraju, A. & Sawyer G.W. (2011). "Comparison of the kinetics and extent of muscarinic M1-M5 receptor internalization, recycling and downregulation in Chinese hamster ovary cells." European Journal of Pharmacology **650**(2-3): 534-543.

Theoharides, T.C., Sant G.R., et al. (1995). "Activation of bladder mast cells in interstitial cystitis: a light and electron microscopic study." Journal of Urology **153**(3 Pt 1): 629-636.

Thind, P., Lose G., et al. (1990). "Variations in urethral and bladder pressure during stress episodes in healthy women." British Journal of Urology **66**(4): 389-392.

Thiyagarajan, M., Kaul C. L., & Ramarao, P. (2002). "Enhancement of α-adrenoceptor-mediated responses in prostate of testosterone-treated rat." European Journal of Pharmacology **453**(2–3): 335-344.

Thonberg, H., Fredriksson J.M., et al. (2002). "A novel pathway for adrenergic stimulation of cAMP-response-element-binding protein (CREB) phosphorylation: mediation via alpha1-adrenoceptors and protein kinase C activation." The Biochemical Journal **364**(Pt 1): 73-79.

Thor, K.B. & Muhlhauser M.A. (1999). "Vesicoanal urethroanal, and urethrovesical reflexes initiated by lower urinary tract irritation in the rat." American Journal of Physiology **277**(4pt2): R1002-R1012.

Thor, K.B. & De Groat W.C. (2010). "Neural control of the female urethral and anal rhabdosphincters and pelvic floor muscles." American Journal of Physiology; Regulatory, Integrative and Comparative Physiology **299**(2): R416-R438.

Thor, K. B., Hisamitsu T. & De Groat W.C. (1990). "Unmasking of a neonatal somatovesical réflex in adult cats by the serotonin autoreceptor agonist 5-methoxy-N,N-dimethyltryptamine." Developmental Brain Research **54**(2): 35-42.

Thor, K. B., Morgant C., et al. (1989). "Organisation of afferent and efferent pathways in the pudendal nerve of the female cat." Journal of Comparative Neurology **288**(2): 263-279.

Thornbury, K.D., Hollywood M.A. & McHale N.G. (1992). "Mediation by nitric oxide of neurogenic relaxation of the urinary bladder neck muscle in sheep." Journal of Physiology **451:** 133-144.

Tichenor, S.D., Buxton I.L., et al. (2002). "Excitatory motor innervation in the canine rectoanal region: role of changing receptor populations." British Journal of Pharmacology **137**(8): 1321-1329.

Timóteo, M.A., Carneiro I., et al. (2014). "ATP released via pannexin-1 hemichannels mediates bladder overactivity triggered by urothelial P2Y6 receptors." Biochemical Pharmacology **87**(2): 371-379.

Tobin, A.B., Lambert D.G. & Nahorski S.R. (1992). "Rapid desensitization of muscarinic m3 receptor-stimulated polyphosphoinositide responses." Molecular Pharmacology **42**(6): 1042-1048.

Tobin, G. & Sjögren C. (1995). "In vivo and in vitro effects of muscarinic receptor antagonists on contractions and release of [3H] acetylcholine in the rabbit urinary bladder." European Journal of Pharmacology **281**(1): 1-8.

Tottrup, A., Glavind E.B. & Svane D. (1992). "Involvement of the L-arginine-nitric oxide pathway in internal anal sphincter relaxation." Gastroenterology **102**(2):409-415.

Townsend, M. K., Matthews C. A., et al. (2013). "Risk factors for fecal incontinence in older women." American Journal of Gastroenterology **108**(1): 113-119.

Tran, T. M., Friedman J., et al. (2004). "Characterization of agonist stimulation of cAMP-dependent protein kinase and G protein-coupled receptor kinase phosphorylation of the beta2-adrenergic receptor using phosphoserine-specific antibodies." Molecular Pharmacology **65**(1): 196-206.

Tran, T. M., Friedman J., et al. (2007). "Characterization of beta2-adrenergic receptor dephosphorylation: Comparison with the rate of resensitization." Molecular Pharmacology **71**(1): 47-60.

Trendelenburg, A.U., Meyer A., et al. (2005). "Distinct mixtures of muscarinic receptor subtypes mediate inhibition of noradrenaline release in different mouse peripheral tissues, as studied with receptor knockout mice." British Journal of Pharmacology **145**(8): 1153-1159.

Trowbridge, E.R., Wei J.T., et al. (2007). "Effects of aging on lower urinary tract and pelvic floor function in nulliparous women." Obstetrics and Gynecology **109**(3): 715-720.

Truschel, S.T., Wang E., et al. (2002). "Stretch-regulated exocytosis/endocytosis in bladder umbrella cells." Molecular Biology of the Cell **13**(3): 830-846.

Tsai, M.H., Kamm K.E. & Stull J.T. (2012). "Signalling to contractile proteins by muscarinic and purinergic pathways in neurally stimulated bladder smooth muscle." Journal of Physiology **590**: 5107–5121.

Tsai, M.H., Chang A.N., et al. (2014). "Constitutive phosphorylation of myosin phosphatase targeting subunit-1 in smooth muscle." Journal of Physiology **592**(14): 3031-3051.

Tsuga, H., Kameyama K., et al. (1994). "Sequestration of muscarinic acetylcholine receptor m2 subtypes. Facilitation by G protein-coupled receptor kinase (GRK2) and attenuation by a dominant-negative mutant of GRK2." Journal of Biological Chemistry **269**(51): 32522-32527.

Tsuga, H., Okuno E., et al. (1998). "Sequestration of human muscarinic acetylcholine receptor hm1-hm5 subtypes: effect of G protein-coupled receptor kinases GRK2, GRK4, GRK5 and GRK6." Journal of Pharmacology and Experimental Therapeutics **284**(3): 1218-1226.

Tsujii, T., Azuma H., et al. (1992). "A possible role of decreased relaxation mediated by beta-adrenoceptors in bladder outlet obstruction by benign prostatic hyperplasia." British Journal of Pharmacology **107**(3): 803-807.

Tunn, R., Goldammer K. et al. (2006). MRI morphology of the levator ani muscle, endopelvic fascia, and urethra in women with stress urinary incontinence. European Journal of Obstetrics, Gynecology and Reproductive Biology **126**(2):239-245.

Tyagi, S., Tyagi P., et al. (2006). "Qualitative and Quantitative Expression Profile of Muscarinic Receptors in Human Urothelium and Detrusor." Journal of Urology **176**(14pt1): 1673-1678.

Tyagi, P., Thomas C.A., et al. (2009). "Investigations into the presence of functional Beta1, Beta2 and Beta3-adrenoceptors in urothelium and detrusor of human bladder." International Brazilian Journal of Urology **35**(1): 76-83.

Tykocki, N.R., Heppner T.J. & Nelson M.T. (2013). "TRPV1-dependent afferent nerve sensitivity is augmented during spontaneous phasic contractions in aged mouse urinary bladder." FASEB Journal **27**: lb868.

Uberti, M.A., Hall R.A. & Minneman K.P. (2003). "Subtype-specific dimerization of alpha 1-adrenoceptors: effects on receptor expression and pharmacological properties." Molecular Pharmacology **64**(6): 1379-1390.

Uberti, M.A., Hague C., et al. (2005). "Heterodimerization with beta2-adrenergic receptors promotes surface expression and functional activity of alpha1D-adrenergic receptors." Journal of Pharamacology and Experimental Therapeutics **313**(1): 16-23.

Uchida, H., Shishido K., et al. (2005). "Involvement of cyclic AMP-dependent and -independent mechanisms in the relaxation of rat detrusor muscle via beta-adrenoceptors." European Journal of Pharmacology **518**(2-3): 195–202.

Uckert, S., Sormes M., et al. (2008). "Effects of phosphodiesterase inhibitors on tension induced by norepinephrine and accumulation of cyclic nucleotides in isolated human prostatic tissue." Urology **71**(3): 526-530.

Uddman, R., Alumets J. et al. (1978). "Peptidergic (VIP) innervation of the esophagus." Gastroenterology **75**(1):5-8.

Uehata, M., Ishizaki T., et al. (1997). "Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension." Nature **389**(6654): 990-994.

Vaizey, C.J., Kamm M.A. &Bartram C.I. (1997). Primary degeneration of the internal anal sphincter as a cause of passive faecal incontinence. Lancet **349**(9052): 612–615.

Valet, P., Senard J.M., et al. (1993). "Characterization and distribution of alpha 2-adrenergic receptors in the human intestinal mucosa." Journal of Clinical Investigation **91**(5): 2049-2057.

Van Geldre, L.A. & Lefebvre R.A. (2004). "Interaction of NO and VIP in gastrointestinal smooth muscle relaxation." Current Pharmaceutical Design **10**(20): 2483-2497.

Vatner, D.E., Lavallee M., et al. (1985). "Mechanisms of supersensitivity to sympathomimetic amines in the chronically denervated heart of the conscious dog." Circulation Research **57**(1): 55–64.

Vázquez-Prado, J., Medina L.C & García-Sáinz J.A. (1997). "Activation of endothelin ETA receptors induces phosphorylation of alpha1b-adrenoreceptors in Rat-1 fibroblasts." Journal of Biological Chemistry **272**(43): 27330-27337.

Vázquez-Prado, J., Medina L.C., et al. (2000). "Norepinephrine- and phorbol ester-induced phosphorylation of alpha(1a)-adrenergic receptors. Functional aspects." Journal of Biological Chemistry **275**(9): 6553-6559.

Veenboer, P.W. & Bosch J.L. (2014). "Long-term adherence to antimuscarinic therapy in everyday practice: a systematic review." Journal of Urology **191**(4): 1003-1008.

Vella M., Duckett J. & Basu M. (2008). "Duloxetine 1 year on: the long-term outcome of a cohort of women prescribed duloxetine." International Urogynecology Journal and pelvic Floor Dysfunction **19**(7): 961-964.

Venkova K. & Krier J. (1993) Stimulation of lumbar sympathetic nerves evokes contractions of cat colon circular muscle mediated by vrydagATP and noradrenaline. British Journal of Pharmacology **110**(3):1260-1270.

Verma, A., Hirsch D.J. et al. (1993). "Carbon monoxide: a putative neural messenger." Science **259**(5093):381-384.

Vesela, R., Asklund H., et al. (2012). "Coupled nitric oxide and autonomic receptor functional responses in the normal and inflamed urinary bladder of the rat." Physiological Research **61**(4): 371-380.

Vial, C. & Evans R.J. (2000). "P2X receptor expression in mouse urinary bladder and the requirement of P2X(1) receptors for functional P2X receptor responses in the mouse urinary bladder smooth muscle." British Journal of Pharmacology **131**(7): 1489-1495.

Vinge, L.E., Øie E., et al. (2001). "Myocardial distribution and regulation of GRK and beta-arrestin isoforms in congestive heart failure in rats." American Journal of Physiology; Heart and circulatory Physiology **281**(6): H2490-H2499.

Vinge, L.E., Andressen K.W., et al. (2007). "Substrate specificities of g protein-coupled receptor kinase-2 and -3 at cardiac myocyte receptors provide basis for distinct roles in regulation of myocardial function." Molecular Pharmacology **72**(3): 582-591.

Viramontes, B.E., Malcolm A., et al. (2001). "Effects of an alpha(2)-adrenergic agonist on gastrointestinal transit, colonic motility, and sensation in humans." American Journal of Physiology; Gastrointestinal and Liver Physiology **281**(6): G1468-G1476.

Vitton, V., Soudan D., et al. (2014). "Treatments of faecal incontinence: recommendations from the French national society of coloproctology." Colorectal Disease **16**(3): 159-166.

Vizzard, M.A. (2000). "Up-regulation of pituitary adenylate cyclase-activating polypeptide in urinary bladder pathways after chronic cystitis." The Journal of Comparative Neurology **420**(3): 335-348.

Vizzard, M.A., Erdman S.L., et al. (1994). "Differential distribution of nitric oxide synthase in neural pathways to the urogenital organs (urethra, penis, urinary bladder) of the rat." Brain Research **646**(2): 279-291.

Vizzard, M.A., Erdman S.L., et al. (1995a). "Increased expression of neuronal nitric oxide synthase in dorsal root ganglion neurons after systemic capsaicin administration." Neuroscience **67**(1): 1-5.

Vizzard, M.A., Erickson V.L., et al. (1995b). "Transneuronal labeling of neurons in the adult rat brainstem and spinal cord after injection of pseudorabies virus into the urethra." The Journal of Comparative Neurology **355**(4): 629-640.

Vizzard, M.A., Erdman S.L., et al. (1996). "Increased expression of neuronal nitric oxide synthase in bladder afferent pathways following chronic bladder irritation." The Journal of Comparative Neurology **370**(2): 191-202.

Vogler, O., Bogatkewitsch G.S., et al. (1998). "Receptor subtype-specific regulation of muscarinic acetylcholine receptor sequestration by dynamin. Distinct sequestration of m2 receptors." Journal of Biological Chemistry **274**(18): 12333-12338.

Vogler, O., Nolte B., et al. (1999). "Regulation of muscarinic acetylcholine receptor sequestration and function by beta-arrestin." Journal of Biological Chemistry **273**(20): 12155-12160.

Vogalis, F.& Goyal R.K. (1997). Activation of small conductance Ca(2+)-dependent K+ channels by purinergic agonists in smooth muscle cells of the mouse ileum. Journal of Physiology **502**(pt3): 497–508.

Vrydag, W., Alewijnse A. E., & Michel M. C. (2009). "Do gene polymorphisms alone or in combination affect the function of human beta3-adrenoceptors?" British Journal of Pharmacology **156**(1): 127-134.

Wagg, A., Cardozo L., et al. (2014). "The efficacy and tolerability of the ß3-adrenoceptor agonist mirabegron for the treatment of symptoms of overactive bladder in older patients." Age and Ageing **43**(5): 666-675.

Wakabayashi, Y., Tomoyoshi T., et al. (1993). "Substance P-containing axon terminals in the mucosa of the human urinary bladder: pre-embedding immunohistochemistry using cryostat sections for electron microscopy." Histochemistry **100**(6): 401-407.

Walden, P. D., Durkin M. M., et al. (1997). "Localization of mRNA and receptor binding sites for the alpha 1a-adrenoceptor subtype in the rat, monkey and human urinary bladder and prostate." Journal of Urology **157**(3): 1032-1038.

Walsh, M. P., Thornbury K., et al. (2011). "Rho-associated kinase plays a role in rabbit urethral smooth muscle contraction, but not via enhanced myosin light chain phosphorylation." American Journal of Physiology - Renal Physiology **300**(1): F73-F85.

Walters, R.D., McMurray G. & Brading A.F. (2006). "Comparison of the urethral properties of the female guinea pig and rat." Neurourology and Urodynamics **25**(1): 62-9.

Wan, M., Zhang W. et al. (2015). Unraveling a molecular determinant for clathrin-independent internalization of the M2 muscarinic acetylcholine receptor. Scientific Reports **5**: 11408.

Wang, R. & Wu L. (1997). "The chemical modification of KCa channels by carbon monoxide in vascular smooth muscle cells." Journal of Biological Chemistry **272**(13):8222-8226.

Wang, P., Luthin G.R. & Ruggieri M.R. (1995). "Muscarinic acetylcholine receptor subtypes mediating urinary bladder contractility and coupling to GTP binding proteins." Journal of Pharmacology and Experimental Therapeutics **273**(2): 959-966.

Wang, R., Wu L. & Wang Z. (1997). "The direct effect of carbon monoxide on KCa channels in vascular smooth muscle cells." Pfluger's Archives: European Journal of Physiology 434(3):285-291.

Wang, E.C., Lee J.M., et al. (2005). "ATP and purinergic receptor–dependent membrane traffic in bladder umbrella cells." A Journal of Clinical Investigation **115**(9): 2412–2422.

Wang, S.Y., Song Y., et al. (2007). "Internalization and distribution of three alpha1-adrenoceptor subtypes in HEK293A cells before and after agonist stimulation." Acta Pharmacologica Sinica **28**(3): 359-366.

Wang, X., Momota Y., et al. (2008). "Urothelium EP1 receptor facilitates the micturition reflex in mice." Biomedical Research **29**(2): 105-111.

Wang, T., Kendig D.M., et al. (2012). "Phorbol 12,13-dibutyrate-induced, protein kinase C-mediated contraction of rabbit bladder smooth muscle." Frontiers in Pharmacology **2**: 83.

Wang, C., Houghton M.J., et al. (2013). "Changes in the innervation of the mouse internal anal sphincter during aging." Neurogastroenterology and Motility **25**(7): e469-e477.

Warburton, A.L. & Santer R.M. (1994). "Sympathetic and sensory innervation of the urinary tract in young adult and aged rats: a semi-quantitative histochemical and immunohistochemical study." Histochemical Journal **26**(2): 127-133.

Ward, S.M., Xue C., et al. (1992). "NADPH diaphorase and nitric oxide synthase colocalization in enteric neurons of canine proximal colon." American Journal of Physiology **263**(2 Pt 1): G277-G284.

Watkins, C.C., Boehning D., et al. (2004). "Carbon monoxide mediates vasoactive intestinal polypeptide-associated nonadrenergic/noncholinergic neurotransmission." Proceedings of National Academy of Sciences of the U.S.A. **101**(8): 2631–2635.

Waugh, M. G., John Challiss, et al. (1999). "Agonist-induced desensitization and phosphorylation of m1-muscarinic receptors." Biochemical Journal **338**(1): 175-183.

Wegener, J.W., Loga F., et al. (2014). "Phospholipase D1 is involved in a1-adrenergic contraction of murine vascular smooth muscle." FASEB Journal **28**(3): 1044-1048.

Wei, H.-B., Yamamura H. I., & Roeske, W. R. (1994). "Down-regulation and desensitization of the muscarinic M1 and M2 receptors in transfected fibroblast B82 cells." European Journal of Pharmacology: Molecular Pharmacology **268**(3): 381-391.

Weigl, L., Zidar A., et al. (2003). "Store operated Ca2+ influx by selective depletion of ryanodine sensitive Ca2+ pools in primary human skeletal muscle cells." Naunyn-Schmiedeberg's Archives of Pharmacology **367**(4): 353-363.

Werkstrom, V., & Andersson K. E. (2005). "ATP- and adenosine-induced relaxation of the smooth muscle of the pig urethra." British Journal of Urology International **96**(9): 1386-1391.

Werkstrom, V., Persson K., et al. (1995). "Factors involved in the relaxation of female pig urethra evoked by electrical field stimulation." British Journal of Pharmacology **116**(1): 1599-1604.

Wessler, I., Reinheimer T., et al. (1994). "Beta-adrenoceptors mediate inhibition of [3H]-acetylcholine release from the isolated rat and guinea-pig trachea: role of the airway mucosa and prostaglandins." British Journal of Pharmacology **113**(4): 1221-1230.

Wheeler, M.A., Hausladen D.A., et al. (2002). "Prostaglandin E2 production and cyclooxygenase-2 induction in human urinary tract infections and bladder cancer." Journal of Urology **168**(4 Pt 1): 1568-1573.

Whisler, R.L. & Grants I.S. (1993). "Age-related alterations in the activation and expression of phosphotyrosine kinases and protein kinase C (PKC) among human B cells." Mechanism of Ageing and Developement **71**(1-2): 31-46.

White, R.A. & Pomeroy C.J. (1989). "Phenylpropanolamine: an alpha-adrenergic agent for the management of urinary incontinence in the bitch associated with urethral sphincter mechanism incompetence." The Veterinary Record **125**(19): 478-480.

Whitehead, W.E., Borrud L., et al. (2009). "Fecal incontinence in US adults: epidemiology and risk factors." Gastroeneterology **137**(2): 512-517, e1-2.

Williams, B.R., Barber R. & Clark R.B. (2000). "Kinetic analysis of agonist-induced down-regulation of the beta(2)-adrenergic receptor in BEAS-2B cells reveals high- and low-affinity components." Molecular Pharmacology **58**(2): 421-430.

Wilson, L., Brown J.S., et al. (2001). "Annual direct cost of urinary incontinence." Obstetrics and Gynecology **98**(3): 398-406.

Winstel, R., Freund S., et al. (1996). "Protein kinase cross-talk: Membrane targeting of the β-adrenergic receptor kinase by protein kinase C." Proceedings of National Academy of Science of the U. S .A. **93**(5): 2105-2109.

Wiseman, O.J., Brady C.M., et al. (2002). "The ultrastructure of bladder lamina propria nerves in healthy subjects and patients with detrusor hyperreflexia." Journal of Urology **168**(5): 2040-2045.

Wiseman, O.J., Fowler C.J. & Landon D.N. (2003). "The role of the human bladder lamina propria myofibroblast." British Journal of Urology International **91**(1): 89-33.

Witte, L.P.W., De Haas N., et al. (2011). "Muscarinic receptor subtypes and signalling involved in the attenuation of isoprenaline-induced rat urinary bladder relaxation." Naunyn-Schmiedeberg's Archive of Pharmacology **384**: 555–563.

Woock, J.P., Yoo P.B. & Grill W.M. (2009). "Intraurethral stimulation evokes bladder responses via 2 distinct reflex pathways." Journal of Urology **182**(1): 366-373.

Wood, J.D. (2010). "Enteric nervous system: sensory physiology, diarrhea and constipation." Current opinions in Gastroenterology **26**(2): 102-108.

Wright, C.D., Chen Q., et al. (2008). “Nuclear alpha1-adrenergic receptors signal activated ERK localization to caveolae in adult cardiac myocytes.” Circulation Research **103**(9): 992-1000.

Wu, C., Sui G. & Fry C.H. (2002). “The role of the L-type Ca(2+) channel in refilling functional intracellular Ca(2+) stores in guinea-pig detrusor smooth muscle.” Journal of Physiology **538**(Pt 2): 357-369.

Wu, C., Sui G.P. & Fry C.H. (2004). “Purinergic regulation of guinea pig suburothelial myofibroblasts.” Journal of Physiology **559**: 231–243.

Wu, D., Katz A., et al. (1992). “Activation of phospholipase C by alpha 1-adrenergic receptors is mediated by the alpha subunits of Gq family.” Journal of Biological Chemistry **267**(36): 25798-25802.

Wu, X., Kushwaha N., et al. (2013). “Role of protein kinase C in agonist-induced desensitization of 5-HT1A receptor coupling to calcium channels in F11 cells.” European Journal of Pharmacology **706**(1–3): 84-91.

Wuest, M., Eichhorn B., et al. (2008). “Muscarinic receptor expression and receptor-mediated detrusor contraction: comparison of juvenile and adult porcine tissue.” Pfugers Archives **456**(2): 349-358.

Wyndaele, J.J. & Van Dromme S.A. (2002). Muscular weakness as side effect of botulinum toxin injection for neurogenic detrusor overactivity. Spinal Cord **40**(11): 599-600.

Xiang, B., Yu G.H., et al. (2001). “Heterologous activation of protein kinase C stimulates phosphorylation of delta-opioid receptor at serine 344, resulting in beta-arrestin- and clathrin-mediated receptor internalization.” Journal of Biological Chemistry **276**(7): 4709-4716.

Xiang, Y., Rybin V.O., et al. (2002). “Caveolar localization dictates physiologic signaling of beta 2-adrenoceptors in neonatal cardiac myocytes.” Journal of Biological Chemistry **277**(37): 34280-34286.

Xiao, A., Wang H., et al. (2015). “H2S, a novel gasotransmitter, involves in gastric accommodation.” Scientific Reports **5**: 16086.

Xing, M. & Insel P.A. (1996). “Protein kinase C-dependent activation of cytosolic phospholipase A2 and mitogen-activated protein kinase by alpha 1-adrenergic receptors in Madin-Darby canine kidney cells.” Journal of Clinical Investigation **97**(5): 1302-1310.

Xu, S., Li X., et al. (2015). “A Method for Recording Urethral Pressure Profiles in Female Rats.” Plose One **10**(10): e0140851.

Xu, X., Menees S.B., et al. (2012). “Economic cost of fecal incontinence.” Diseases of the Colon and Rectum **55**(5): 586-598.

Xue, L., Farrugia G., et al. (2000). "Carbon monoxide and nitric oxide as coneurotransmitters in the enteric nervous system: evidence from genomic deletion of biosynthetic enzymes." Proceedings of the National Academy of Science of the United States of America **97**(4): 1851-1855.

Yamaguchi, O. (2010). "Antimuscarinics and overactive bladder: other mechanism of action." Neurology and Urodynamics **29**(1): 112-115.

Yamaguchi O., Marui E., et al. (2014). "Phase III, randomised, double-blind, placebo-controlled study of the ß3-adrenoceptor agonist mirabegron, 50mg once daily, in Japanese patients with overactive bladder." British Journal of urology **113**(6): 951-960.

Yamanishi T., Chapple C.R., et al. (2000). The role of M(2)-muscarinic receptors in mediating contraction of the pig urinary bladder in vitro. British Journal of Pharmacology **131**(7):1482-1488.

Yamanishi T., Chapple C.R. & Yasuda K. (2002a). "The role of M2 muscarinic receptor subtypes in mediating contraction of the pig bladder base after cyclic adenosine monophosphate elevation and/or selective M3 inactivation." Journal Urology **167**: 397–401.

Yamanishi, T., Chapple C.R., et al. (2002b). "The role of beta(3)-adrenoceptors in mediating relaxation of porcine detrusor muscle." British Journal of Pharmacology **135**(1): 129-134.

Yamanishi, T., Chapple C.R., et al. (2002c). "Identification of beta-adrenoceptor subtypes in lower urinary tract of the female pig." Journal of Urology **168**(6): 2706-1710.

Yamanishi, T., Chapple C.R., et al. (2002d). "The role of M2 muscarinic receptor subtypes mediating contraction of the circular and longitudinal smooth muscle of the pig proximal urethra." Journal of Urology **168**(1): 308-314.

Yamanishi, T., Chapple C.R., et al. (2003a). "Role of beta-adrenoceptor subtypes in mediating relaxation of the pig bladder trigonal muscle in vitro." Neurourology and Urodynamics **22**(4): 338-42.

Yamanishi, T., Chapple C.R., et al. (2003b). "The functional role of beta-adrenoceptor subtypes in mediating relaxation of pig urethral smooth muscle." Journal of Urology **170**(6 Pt 1): 2508-2511.

Yamato, S. & Rattan S. (1990). "Role of alpha adrenoceptors in opossum internal anal sphincter." Journal of Clinical Investigation **86**(2): 424-429.

Yanai, Y., Hashitani H., et al. (2006). "The role of Ni(2+)-sensitive T-type Ca(2+) channels in the regulation of spontaneous excitation in detrusor smooth muscles of the guinea-pig bladder." Britsih Journal of Urology International **97**(1): 182-189.

Yanase, H., Wang X., et al. (2008). "The involvement of urothelial alpha1A adrenergic receptor in controlling the micturition reflex." Biomedical Research **29**(5): 239-244.

Yang, J., Sun H., et al. (2015). "Regulation of ß-adrenergic receptor trafficking and lung microvascular endothelial cell permeability by Rab5 GTPase." International Journal of Biological Sciences **11**(8): 868-878.

Yao, Y., Bergold P.J. & Penington N.J. (2010). "Acute Ca(2+)-dependent desensitization of 5-HT(1A) receptors is mediated by activation of protein kinase A (PKA) in rat serotonergic neurons." Neuroscience **169**(1): 87-97.

Yeatman, H.R., Lane J.R., et al. (2014). "Allosteric modulation of M1 muscarinic acetylcholine receptor internalization and subcellular trafficking." Journal of Biological Chemistry **289**(22): 15856-15866.

Yiangou, Y., Facer P., et al. (2001). "Capsaicin receptor VR1 and ATP-gated ion channel P2X3 in human urinary bladder." Bristish Journal of Urology **87**(9): 774-779.

Yin, Q., Yang C., et al. (2016). "Downregulation of ß-Adrenoceptors in Isoproterenol-Induced Cardiac Remodeling through HuR." Plos One **11**(4): e0152005.

Yip, S.O., Dick M.A., et al. (2013). "The association between urinary and fecal incontinence and social isolation in older women." American Journal of Obstetrics and Gynecology **208**(2): 146.e1-e7.

Yono, M., Foster, H. E. Jr., et al. (2004). "Doxazosin-induced up-regulation of alpha 1A-adrenoceptor mRNA in the rat lower urinary tract." Canadian Journal of Physiology and Pharmacology **82**(10): 872-878.

Yono, M., Foster H.E. Jr., et al. (2006). "Age related changes in the functional, biochemical and molecular properties of alpha1-adrenoceptors in the rat genitourinary tract." Journal of Urology **176**(3): 1214-1219.

Yoshida, M., Inadome A., et al. (2006). "Non-neuronal cholinergic system in human bladder urothelium." Urology **67**: 425–430.

Yoshida, M., Masunaga K., et al. (2008). "Basic and clinical aspects of non-neuronal acetylcholine: expression of non-neuronal acetylcholine in urothelium and its clinical significance." Journal of Pharmaceutical Sciences **106**: 193–198.

Yoshida, N., Jojima E., et al. (2014). "Role of the third intracellular loop in the subtype-specific internalization and recycling of muscarinic M2 and M4 receptors." Biomedical Research **35**(3): 185-192.

Yoshiki, H., Uwada J., et al. (2013). "Agonist pharmacology at recombinant a1A - and a1L - adrenoceptors and in lower urinary tract a1 -adrenoceptors." British Journal of Pharmacology **170**(6): 1242-1252.

Yoshimura, N., Seki S., et al. (2003). "Histological and electrical properties of rat dorsal root ganglion neurons innervating the lower urinary tract." Journal of Neuroscience **23**(10): 4355-4361.

Yoshimura, N. & Miyazato M. (2012). “Neurophysiology and therapeutic receptor targets for stress urinary incontinence.” International Journal of Urology **19**(6): 524-537.

Young, J.S., Matharu R., et al. (2012). “Inhibition of stretching-evoked ATP release from bladder mucosa by anticholinergic agents.” British Journal of Urology International **110**(8 Pt B): E397-E401.

Yousufzai, S.Y., & Abdel-Latif, A.A. (1983). “Effects of norepinephrine and other pharmacological agents on prostaglandin E2 release by rabbit and bovine irides.” Experimental eye research **37**(3): 279-292.

Yousufzai, S.Y., & Abdel-Latif, A.A. (1984). “The effects of alpha 1-adrenergic and muscarinic cholinergic stimulation on prostaglandin release by rabbit iris.” Prostaglandins **28**(3): 399-415.

Yu, Y. & De Groat W.C. (2008). “Sensitization of pelvic afferent nerves in the in vitro rat urinary bladder-pelvic nerve preparation by purinergic agonists and cyclophosphamide pretreatment.” American Journal of Physiology; Renal Physiology **294**(5): F1146-F1156.

Yu, W., Hill W.G., et al. (2011). “Expression and distribution of transient receptor potential (TRP) channels in bladder epithelium.” American Journal of Physiology; Renal Physiology **300**(1): F49-F59.

Yu, W., Sun X., et al. (2013). “Extracellular UDP enhances P2X-mediated bladder smooth muscle contractility via P2Y (6) activation of the phospholipase C/inositol trisphosphate pathway.” FASEB Journal **27**(5): 1895-1903.

Yucel, S. & Baskin L.S. (2004). “An anatomical description of the male and female urethral sphincter complex.” Journal of Urology **171**(5): 1890-1897.

Zaghiyan, K.N. & Fleshner P. (2011). “Anal fissure.” Clinics in Colon and Rectal Surgery **24**(1): 22-30.

Zagorodnyuk, V.P., Lynn P., et al. (2005). “Mechanisms of mechanotransduction by specialized low-threshold mechanoreceptors in the guinea pig rectum.” American Journal of Physiology; Gastrointestinal and Liver Physiology **289**: G397–G406.

Zagorodnyuk, V.P., Costa M. & Brookes S.J. (2006). “Major classes of sensory neurons to the urinary bladder.” Autonomic Neuroscience **126-127**: 390-97.

Zagorodnyuk, V.P., Gibbins I.L. et al. (2007). “Properties of the major classes of mechanoreceptors in the guinea pig bladder.” Journal of Physiology **585** (Pt1): 147-163.

Zagorodnyuk, V.P., Gregory S., et al. (2009). “Spontaneous release of acetylcholine from autonomic nerves in the bladder.” British Journal of Pharmacology **157**(4): 607-619.

Zakhary, R., Poss K.D., et al. (1997). "Targeted gene deletion of heme oxygenase 2 reveals neural role for carbon monoxide." Proceedings of National Science Academy of the United States of America **94**(26): 14848-14853.

Zamah, A.M., Delahunty M., et al. (2002). "Protein kinase A-mediated phosphorylation of the beta 2-adrenergic receptor regulates its coupling to Gs and Gi. Demonstration in a reconstituted system." Journal of Biological Chemistry **277**(34): 31249-31256.

Zarghooni, S., Wunsch J., et al. (2007). "Expression of muscarinic and nicotinic acetylcholine receptors in the mouse urothelium." Life Sciences **80**(24-25): 2308-2313.

Zhang, Y. & Hu W. (2013). "Mouse enteric neuronal cell culture." Methods in Molecular Biology **1078**:55-63.

Zhang, L.B., Horowitz B. & Buxton I.L. (1991). "Muscarinic receptors in canine colonic circular smooth muscle. I. Coexistence of M2 and M3 subtypes." Molecular Pharmacology **40**(6): 943-951.

Zhang, L., Keef K.D., et al. (1992). "Action of alpha 2A-adrenergic receptors in circular smooth muscle of canine proximal colon." American Journal of Physiology **262**(3 Pt 1): G517-G524.

Zhang, X.Y., Olszewski M.A., et al. (1995). "Beta 2-adrenoceptor activation augments acetylcholine release from tracheal parasympathetic nerves." American Journal of Physiology **268**(6 Pt 1): L950-L956.

Zhang, X.Y., Zhu F.X., et al. (1998). "Effects of enantiomers of beta 2-agonists on ACh release and smooth muscle contraction in the trachea." American Journal of Physiology **274**(1 Pt 1): L32-L38.

Zhang, S., Zheng S., et al. (2015). "Carbon Monoxide-Releasing Molecule-2 Reduces Intestinal Epithelial Tight-Junction Damage and Mortality in Septic Rats." Plos One **10**(12): e0145988.

Zhang, X., Alwaal A., et al. (2016a). "Urethral musculature and innervation in the female rat." Neurourology and Urodynamics **35**(3): 382-389.

Zhang, C.H., Wang P., et al. (2016b). "The molecular basis of the genesis of basal tone in internal anal sphincter." Nature communication **7**: 11358.

Zhao W., Zhang J., et al. (2001). The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. EMBO Journal **20**(21): 6008–6016.

Zhao, W., Aboushwareb T., et al. (2010). "Impaired bladder function in aging male rats." Journal of Urology **184**(1): 378-385.

Zhao, Y., Vanhoutte P.M., et al. (2015). “a1 -Adrenoceptor activation of PKC-e causes heterologous desensitization of thromboxane receptors in the aorta of spontaneously hypertensive rats.” British Journal of Pharmacology **172**(14): 3687-3701.

Zhou, L., & Vargas H. M. (1996). “Vascular alpha 1D-adrenoceptors have a role in the pressor response to phenylephrine in the pithed rat.” European Journal of Pharmacology **305**(1-3): 173-176.

Zhu, L., Lang J., et al. (2009). The epidemiological study of women with urinary incontinence and risk factors for stress urinary incontinence in China. Menopause **16**(4): 831-836.

Zyczynski, H.M., Albo M.E. et al. (2015). Change in Overactive Bladder Symptoms After Surgery for Stress Urinary Incontinence in Women. Obstetrics and Gynecology **126**(2): 423-430.

11 APPENDIX

11.1DRUGS, CONCENTRATIONS AND SOLVENTS

Drugs	Concentration used	Mwt	Solvent
8-phenylltheophylline	10μM	256.3	dH_20
AOA	30μM	109.3	dH_20
Atropine	1μM	676.8	dH_20
Calphostin C	1μM	790.76	70% ethanol
Corticosterone	1μM	346.47	70% ethanol
Cyclopiazonic acid	10μM	336.4	DMSO
Desipramine	1μM	302.84	dH_20
EDTA	3mM	292.24	dH_20
Fasudil	10μM	327.83	dH_20
Guanethidine	10μM	296.39	dH_20
Indomethacin	10μM	357.8	70% ethanol
KCl	60mM	74.55	dH_20
L-NNA	100μM	219.20	dH_20
Nifedipine	100nM-1μM	346.34	DMSO
ODQ	10μM	187.16	70% ethanol
PAG	1mM	113.11	70% ethanol
Phe-Leu-VIP	100nM	3342.24	dH_20
Propranolol	1μM	295.8	dH_20
Suramin	100μM	1519.23	dH_20
TTX	1μM	319.27	dH_20
Y27632	10μM	320.26	dH_20
ZnPPIX	10μM	644.05	70% ethanol
αβ-mATP	10μM	505.2	dH_20

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Three Gaseous Neurotransmitters, Nitric oxide, Carbon Monoxide, and Hydrogen Sulfide, Are Involved in the Neurogenic Relaxation Responses of the Porcine Internal Anal Sphincter

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Abstract

Background/Aims

The internal anal sphincter (IAS) plays an important role in maintaining continence and a number of neurotransmitters are known to regulate IAS tone. The aim of this study was to determine the relative importance of the neurotransmitters involved in the relaxant and contractile responses of the porcine IAS.

Methods

Responses of isolated strips of IAS to electrical field stimulation (EFS) were obtained in the absence and presence of inhibitors of neurotransmitter systems.

Results

Contractile responses of the sphincter to EFS were unaffected by the muscarinic receptor antagonist atropine (1 μM), but were almost completely abolished by the adrenergic neuron blocker guanethidine (10 μM). Contractile responses were also reduced (by 45% at 5 Hz, $P <$

0.01) following desensitisation of purinergic receptors with α,β-methylene-ATP (10 μM). In the presence of guanethidine, atropine, and α,β-methylene-ATP, the remaining relaxatory responses to EFS were examined. These responses were not altered by the cyclooxygenase inhibitor indomethacin (5 μM), the vasoactive intestinal polypeptide receptor antagonist [$_{D}$-p-Cl-Phe^{6},Leu^{17}]-vasoactive intestinal peptide (PheLeu-VIP; 100 nM), or the purinoceptor antagonists 8-phenyltheophyline (P1 receptors) or suramin (P2 receptors). However relaxation responses were reduced by Nω-nitro-$_{L}$-arginine (L-NNA; 100 μM) an inhibitor of nitric oxide synthesis (40-50% reduction), zinc protoprophyrin IX (10 μM) an inhibitor of carbon monoxide synthesis (20-40% reduction), and also propargylglycine (30 μM) and aminooxyacetic acid (30 μM), inhibitors of hydrogen sulphide synthesis (15-20% reduction).

Conclusions

Stimulation of IAS efferent nerves releases excitatory and inhibitory neurotransmitters: noradrenaline is the predominant contractile transmitter with a smaller component from ATP, whilst 3 gases mediate relaxation responses to EFS, with the combined contributions being nitric oxide > carbon monoxide > hydrogen sulfide.

Key Words

Anal sphincter; Carbon monoxide; Hydrogen sulfide; Neurotranmission; Nitric oxide

Introduction

The internal anal sphincter (IAS) plays an important role in maintaining continence contributing up to 85% of resting anal tension[1] and thus it is a potential target for the drug treatment of both anal fissures and fecal incontinence. Fecal incontinence is the uncontrolled passing of fecal material and drugs that increase IAS contractile tone may potentially aid continence, whilst drugs that reduce anal pressure may increase anal blood flow and thereby aid healing of anal fissures.

Both the autonomic and enteric nervous systems influence tone of the IAS. It has been shown histologically that the main autonomic input to the IAS originates in the inferior rectal branches of the pelvic plexus with both tyrosine hydroxylase staining fibers (sympathetic) and nitric oxide (NO) staining fibers (presumed to be parasympathetic) innervating the tissue.[2,3] In functional experiments, noradrenaline is the only neurotransmitter that has been identified as eliciting an excitatory response in the sphincter,[4] while inhibitory roles have been suggested for NO, carbon monoxide (CO) and vasoactive intestinal polypeptide (VIP). It has also been suggested that acetylcholine and adenosine 5'-triphosphate (ATP) may also have roles in the relaxation of the sphincter.[4] Although these neurotransmitters are usually excitatory rather than inhibitory, O'Kelly et al[5] has suggested that stimulation of muscarinic receptors by acetylcholine causes the release of NO and a subsequent relaxation of the IAS.

Previous studies using electrical field stimulation of the IAS have demonstrated the involvement of several neurotransmitters in responses of this tissue. However most of these studies have focused on the relaxation of the sphincter and its relevance to anal fissures, whereas the contractile component, which could be a target for drug development relevant to

fecal incontinence, has yet to be explored to the same extent. It is known that NO plays an important role as an inhibitory neurotransmitter in the IAS[6] and that noradrenaline acting via α_1-adrenoceptors is responsible for contracting the sphincter.[7-9] Furthermore, the relative contribution of each neurotransmitter system to the responses of the IAS has not been established. The aim of the present study was to identify the neurotransmitters released during electrical field stimulation (EFS) of the porcine IAS and determine the relative contributions of each transmitter to the functional responses.

Materials and Methods

Female pig internal anal sphincter (IAS) muscle samples were obtained from a local abattoir. The tissues were immediately placed in Krebs-bicarbonate solution (composition in mmol/L: NaCl 118.4, $NaHCO_3$ 24.9, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4$ 1.15, KH_2PO_4 1.15, and glucose 11.7) at 4ºC. The mucosa and submucosa were removed and the circular muscle of the sphincter, approximately 2.5 cm from the anal opening, was cut into strips (15 × 3 mm). The muscle strips were set up in EZ-Bath tissue baths (GlobalTown Microtechnology, Sarasota, FL, USA) containing Krebs-bicarbonate solution at 37ºC and gassed with 5% CO_2 in oxygen. The muscle strips were attached to isometric force transducers connected to a MacLab recording system (AD Instruments Ltd. Bella Vista, NSW Australia) and the developed tension measured using "CHART" software. Tissues were mounted under 1g resting tension and allowed to equilibrate for 30 minutes during which time they were washed with fresh Krebs-bicarbonate solution every 15 minutes.

At this point the smooth muscle strips were electrically stimulated (40 V and 1 millisecond pulse-width) delivered as 5 second trains every 100 seconds, at a frequency of 5

Hz and then 10 Hz. Initial responses for each frequency were obtained followed by responses in the presence of one of the following drugs which interfere with neurotransmission: Nω-nitro-$_L$-arginine (L-NNA, 100 μM), a NO synthase inhibitor; guanethidine (10 μM), a neurotoxin selective for adrenergic nerves; atropine (1 μM), a muscarinic receptor antagonist; α,β-methylene ATP (10 μM), a potent purinergic agonist that desensitises P2X receptors; indomethacin (5 μM), an inhibitor of cyclooxygenase 1 and 2; 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM), an inhibitor of guanylate cyclase; and [$_D$-p-Cl-Phe^6,Leu^{17}]-vasoactive intestinal peptide (PheLeu-VIP, 100 nM), a vasoactive intestinal polypeptide receptor antagonist. Responses to EFS were repeated in the presence of one of the above antagonists/inhibitors which were equilibrated with tissues for 30 minutes before obtaining responses to EFS. Only one drug was tested on each tissue and control experiments confirmed responses to EFS did not change over this short time period.

1. Drugs Used

Atropine (sulfate salt monohydrate), α,β-methylene ATP (lithium salt), L-NNA (Nω-nitro-$_L$-arginine), indomethacin (1-[p-Chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid), PAG (propargylglycine), AOAA (aminooxyacetic acid), methylene blue hydrate, guanethidine (1-[2-Guanidinoethyl]octahydroazocine monosulfate), PheLeu-VIP ([$_D$-p-Cl-Phe^6,Leu^{17}]-vasoactive intestinal peptide), ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), and tetrodotoxin were obtained from Sigma (Illinois, USA). ZnPPIX (zinc protoporphyrin IX) was obtained from Tocris Bioscience (Ellisville, MO, USA).

2. Statistical Methods

Changes in developed tension were represented as a percentage of the tone of the tissue at the time of stimulation and the mean ± SEM were calculated. The responses seen in the presence of antagonist/inhibitor were compared to the initial responses in the absence of antagonist/inhibitor from the same tissue strip using Student's paired *t* test. A *P*-value of ≤ 0.05 was considered statistically significant.

Results

1. Influence of Frequency of Stimulation

The IAS responded to EFS with a relaxation followed by a frequency-dependent contraction (Fig. 1). At 5 Hz stimulation the relaxation was fast, lasting 2-3 seconds, followed by a contraction of approximately 10 seconds duration. Stimulation at a frequency of 10 Hz produced a contraction that was greatly increased in both size and duration and appeared to mask the relaxation. The responses were neurogenic in origin, and the neurotoxin tetrodotoxin (3 μM, n = 8) completely abolished contractions at both frequencies. Relaxations to EFS were also greatly reduced by tetrodotoxin (3 μM) by 78.3 ± 2.8% and 91.5 ± 1.6% at 5 Hz and 10 Hz respectively (both $P < 0.001$).

2. Contractile Responses to Electrical Field Stimulation

Removal of the adrenergic component with guanethidine (10 μM) almost completely abolished the contraction of the IAS to electrical stimulation at both frequencies (Table 1). Guanethidine reduced the large contractile responses at 5Hz and 10 Hz to contractions that were superimposed on large relaxations and only recovered to 30% and 20% below the

baseline respectively (Fig. 1). Contractile responses were also reduced following desensitization of P2X receptors with the potent purinoceptor agonist α,β-mATP (10 μM). Responses at both frequencies were reduced, but the effect was only statistically significant for responses at 5 Hz (Table 1). In contrast, responses to electrical stimulation were not significantly altered by the presence of the muscarinic receptor antagonist atropine (1 μM; Table 1).

3. Relaxation Responses of the Internal Anal Sphincter

The relaxation responses obtained after removing adrenergic, cholinergic, and purinergic contractions with guanethidine (10 μM), atropine (1 μM), and a,β-mATP (10 μM) were also examined in greater detail to determine which inhibitory neurotransmitters were involved and their relative importance. Under these conditions, relaxations to EFS were unaffected by the cyclooxygenase (COX1/2) inhibitor indomethacin (5 μM, n = 14) or the VIP-receptor antagonist PheLeu-VIP (100 nM, n = 6; Table 2). However, in the presence of the NO synthase inhibitor L-NNA (100 μM), relaxations were reduced by 40-50% (Fig. 2). Inhibition of guanylate cyclase with ODQ (10 μM) produced a greater inhibition than L-NNA, but a combined L-NNA/ODQ treatment did not produce a greater inhibition of relaxation than ODQ alone (Fig. 2).

In another series of experiments the non-nitrergic relaxation remaining in the presence of L-NNA (100 μM) was investigated further. As previously, these relaxation experiments were performed in the presence of guanethidine (10 μM), atropine (1 μM), and a,β-mATP (10 μM) to remove contractile responses. The relaxations obtained in the additional presence of L-NNA (100 μM) were not affected by either the adenosine receptor (P1) antagonist 8-phenyltheophyline (10 μM, n = 5) nor the P2-purinoceptor antagonist suramin (100 μM, n =

5; data not shown). The possible contribution to relaxation by CO was examined using zinc protoporphyrin IX (ZnPPIX, 10 μM), which inhibits the synthesis of CO by heme oxygenase, and the contribution to relaxation by hydrogen sulfide (H_2S) was examined using a combination of propargylglycine (PAG, 1 mM) and aminooxyacetic acid (AOAA, 30 μM) to inhibit H_2S synthesis. The inhibition of either CO synthesis or H_2S synthesis reduced relaxation to EFS by 20-40% and 15-20% respectively (Fig. 3). The inhibition of guanylate cyclase with ODQ reduced relaxations by about 70% and removing H_2S with PAG/AOAA further reduced relaxation responses producing greater inhibition than ODQ alone. In contrast the effects of CO removal with ZnPPIX were not additive with ODQ, the combination of ZnPPIX/ODQ yielding the same reduction in relaxation to EFS as ODQ alone (Fig. 3).

Discussion

In the pig anal sphincter, guanethidine was used to investigate the role of noradrenaline in the contraction of this tissue. Guanethidine is a neurotoxin selective for adrenergic nerves and abolishes adrenergic nerve transmission completely at a concentration of 10μM.[10] Guanethidine (10 μM) depressed contractions completely from a large contraction above the baseline to one that failed to increase to the baseline level following the initial relaxation response, suggesting a prolonged relaxation mechanism. Thus guanethidine inhibited contraction by > 100%, which prevented an accurate calculation of the contribution of noradrenaline to contraction of the IAS. Although the exact contribution of noradrenaline could not be calculated it can be concluded that noradrenaline is the main excitatory neurotransmitter released in the IAS. In previous studies we have shown that these adrenergic responses are mediated via $\alpha_{1A/L}$-adrenoceptors.[9]

In the present study desensitisation of P2X receptors also depressed responses to EFS indicating an excitatory neurotransmitter role for ATP in this tissue. ATP is often released as a co-transmitter from autonomic nerves and is released with noradrenaline in many tissues including the colon[11] and vasculature.[12] These previous studies used the agonist α,β-mATP to desensitize P2X purinergic receptors. This potent agonist was used to desensitise the purinergic receptors of the IAS and it depressed contractile responses to EFS, but the inhibition was significantly less than that produced by guanethidine. The reduction of contraction to EFS by α,β-mATP was greater at 5 Hz stimulation than at 10Hz suggesting that ATP provides a greater contribution to contractions at lower frequencies of stimulation. This idea is supported by the report that the inhibition of ATP breakdown has a greater potentiating effect on the neurogenic responses of visceral and vascular smooth muscle at low frequencies compared to higher frequencies (Kennedy et al., 1996). Thus the present results suggest a role for ATP in the neurogenic contractions of the anal sphincter via P2X receptors, at least at lower frequencies of stimulation. Surprisingly, a previous study suggested that ATP is an inhibitory neurotransmitter in the IAS.[4] In other tissues such as the bladder, ATP is able to induce smooth muscle relaxation via 3 possible mechanisms: (1) directly via P2Y receptors, (2) indirectly by inducing NO release from epithelial cells, and (3) indirectly via P1 receptors after being broken down to adenosine.[13] However, in the current studies the mucosa had been removed before tissues were set up and neither the P2-receptor antagonist suramin, nor the P1-receptor antagonist 8-phenyltheophyline had any effect on relaxations, thus ruling out a role for ATP in relaxation responses of this tissue.

After inhibition of the contractile responses, relaxation responses to EFS were greatly enhanced and the mechanisms involved were investigated in more detail. The NO synthase inhibitor L-NNA (100 μM) reduced the relaxation induced by field stimulation by

approximately 40-60%, suggesting that NO is responsible for approximately half of the relaxation response of the sphincter to nerve stimulation. Also a number of other non-nitrergic inhibitory neurotransmitters have been proposed for the IAS including acetylcholine, VIP, CO, and H_2S. Previous studies have suggested that muscarinic receptor stimulation results in relaxation which appears to be mediated via the release of NO.[5] In the present study muscarinic receptor stimulation with carbachol elicited contractions and no relaxation was observed. Furthermore, atropine had no effect on the excitatory responses to EFS and the inhibitory responses were obtained in the presence of atropine, ruling out a contribution from acetylcholine. In other tissues, such as blood vessels muscarinic receptor stimulation of epithelial cells can release NO to induce relaxation.[14] Similarly, in the urinary bladder, muscarinic receptors are responsible for direct smooth muscle contraction,[15] but they may also stimulate release of NO from urothelial cells.[16-18] In the present study experiments were performed on muscle strips with the mucosa removed to allow neurotransmission to be examined without the complication of epithelial influences and no relaxation responses were observed.

The non-nitrergic relaxation induced by EFS in the presence of L-NNA was also investigated. Neither the VIP receptor antagonist PheLeu-VIP nor the COX1/2 inhibitor indomethacin significantly reduce the non-nitrergic relaxation ruling out a role for VIP or prostaglandins in relaxation of the pig IAS, a conclusion also reached by Acheson et al[19] in the sheep IAS. However the guanylate cyclase inhibitor ODQ[20] did have a significant effect, reducing relaxations by approximately 70%. CO, which mediates smooth muscle relaxation responses via cGMP, is an inhibitory neurotransmitter that plays a role in the relaxation of the opossum sphincter.[21] Using immunocytochemistry in the same animal, the enzyme heme oxygenase (HO), which produces CO, has been found to be present in the myenteric and

submucosal neurones of the IAS as well as in the interstitial cells of Cajal (ICCs) found in this tissue.[22] The non-nitrergic relaxation of the pig IAS to field stimulation was greatly reduced by ODQ which suggests the involvement of CO in the relaxation of this tissue. This was confirmed using ZnPPIX an inhibitor of HO which is responsible for the synthesis of CO. This inhibitor reduced IAS relaxation responses by about 20% and this effect was not additive with ODQ. These data suggest that the ODQ sensitive component of the relaxation is composed of predominantly NO (50%) but with a smaller contribution from CO (20%).

In the presence ODQ, the remaining small, non-NO, and non-CO relaxation must be mediated via a neurotransmitter that is cGMP independent. It has been shown that the enzymes responsible for the production of H_2S are present in the enteric nerves and the myenteric ICCs,[23] both of which can be found in the IAS.[8,24,25] Inhibition of H_2S synthesis with PAG/AOAA depressed relaxation responses to EFS and the depression was additive to that of ODQ and therefore independent of cGMP indicating a small role for H_2S in relaxation of the IAS to EFS.

In conclusion the neurogenic contraction of the porcine IAS can be attributed mainly to noradrenaline and ATP, with both neurotransmitters probably released from adrenergic nerves. The smooth muscle of the IAS responds with contraction to muscarinic receptor stimulation, but neurotransmission, at least in the pig IAS, does not appear to involve acetylcholine. The relaxations of the IAS to electrical stimulation appears to be mediated by the simultaneous release of all three gaseous transmitters with relative contributions NO > CO > H_2S. Non-adrenergic non-cholinergic relaxations of the IAS have been shown in other species, with an involvement of NO, CO, and possibly H_2S. However, this study

demonstrates the simultaneous release of all three gaseous neurotransmitters, and their combined contributions, in mediating relaxation of the porcine IAS.

All these transmitters represent possible targets for drug development where enhancing sphincter tone may aid the treatment of fecal incontinence or reductions in IAS tone can aid healing of anal fissures.

References

1. Lestar B, Pennickx F, Kerremans R. The composition of anal basal pressure. An in vivo and in vitro study in man. Int J Colorectal Dis 1989;4:118-122.
2. Kinugasa Y, Arakawa T, Murakami G, Fujimiya M, Sugihara K. Nerve supply to the internal anal sphincter differs from that to the distal rectum: an immunohistochemical study of cadavers. Int J Colorectal Dis 2014;29:429-436.
3. Ishiyama G, Hinata N, Kinugasa Y, Murakami G, Fujimiya M. Nerves supplying the internal anal sphincter: an immunohistochemical study using donated elderly cadavers. Surg Radiol Anat 2014; 36:1033-1042.
4. Rattan. S. The internal anal sphincter: regulation of smooth muscle tone and relaxation. Neurogastroenterol Motil 2005;17(suppl 1):50-59.
5. O'Kelly T, Brading A, Mortensen N. Nerve mediated relaxation of the human internal anal sphincter: the role of nitric oxide. Gut 1993;34:689-693.
6. Rattan S, Regan RF, Patel CA, De Godoy MA. Nitric oxide not carbon monoxide mediates nonadrenergic noncholinergic relaxation in the murine internal anal sphincter. Gastroenterology 2005;129:1954-1966.

7. Matsufuji H, Yokoyama J. Neural control of the internal anal sphincter motility. J Smooth Muscle Res 2003:39:11-20.
8. Yamato S, Rattan S. Role of alpha adrenoceptors in opossum internal anal sphincter. J Clin Invest 1990;86:424-429.
9. Mills K, Hausman N, Chess-Williams R. Characterization of the alpha1-adrenoceptor subtype mediating contractions of the pig internal anal sphincter. Br J Pharmacol 2008;155:110-117.
10. Cobine CA, Fong M, Hamilton R, Keef KD. Species dependent differences in the actions of sympathetic nerves and noradrenaline in the internal anal sphincter. Neurogastroenterol Motil 2007;19:937-945.
11. Venkova K, Krier J. Stimulation of lumbar sympathetic nerves evokes contractions of cat colon circular muscle mediated by ATP and noradrenaline. Br J Pharmacol 1993;110:1260-1270.
12. Racchi H, Irarrázabal MJ, Howard M, Morán S, Zalaquett R, Huidoro-Toro JP. Adenosine 5'-triphosphate and neuropeptide Y are co-transmitters in conjunction with noradrenaline in the human saphenous vein. Br J Pharmacol 1999;126:1175-1185.
13. Burnstock G. Purinergic signalling in the urinary tract in health and disease. Purinergic signal 2014;10:103-155.
14. Khurana S, Chacon I, Xie G, et al. Vasodilatory effects of cholinergic agonists are greatly diminished in aorta from M3R-/- mice. Eur J Pharmacol 2004;493:127-132.
15. Hedge SS. Muscarinic receptors in the bladder: from basic research to therapeutics. Br J Pharmacol 2006;147(suppl 2):S80-S87.

16. Kullmann FA, Artim D, Beckel J, Barrick S, De Groat WC, Birder LA. Heterogeneity of muscarinic receptor-mediated Ca^{2+} responses in cultured urothelial cells from rat. Am J Physiol Renal Physiol 2004;294:F971-F981.
17. Hanna-Mitchell AT, Birder LA. New insights into the pharmacology of the bladder. Current Opin Urol 2008;18:347-352.
18. Andersson MC, Tobin G, Giglio D. Cholinergic nitric oxide release from the urinary bladder mucosa in cyclophosphamide- induced cystitis of the anaesthetized rat. Br J Pharmacol 2008;153:1438-1444.
19. Acheson A, Rayment S, Eames T, et al. Investigation of the role of adrenergic and non-nitrergic, non-adrenergic neurotransmission in the sheep isolated internal anal sphincter. Neurogastroenterol Motil 2009;21:335-345.
20. Dellabianca A, Sacchi M, Anselmi L, et al. Role of carbon monoxide in electrically induced non-adrenergic, non-cholinergic relaxations in the guinea-pig isolated whole trachea. Br J Pharmacol 2007;150:220-226.
21. Rattan S, Al Haj R, De Godoy MA. Mechanism of internal anal sphincter relaxation by CORM-1, authentic CO, and NANC nerve stimulation. Am J Physiol Gastrointest Liver Physiol 2004;287:G605-G611.
22. Battish R, Cao GY, Lynn RB, Chakder S, Rattan S. Heme oxygenase-2 distribution in anorectum: colocalization with neuronal nitric oxide synthase. Am J Physiol Gastrointest Liver Physiol 2000;278:G148-G155.
23. Schicho R, Krueger D, Zeller F, et al. Hydrogen sulphide is a novel prosecretory neuromodulator in the Guinea-pig and human colon. Gastroenterology 2006;131:1542-1552.

24. Shafik A, El Sibai O, Ahmed I. The identification of specialized pacemaking cells in the anal sphincter. Int J Colorectal Dis 2006;21:453-457.

Figure legends

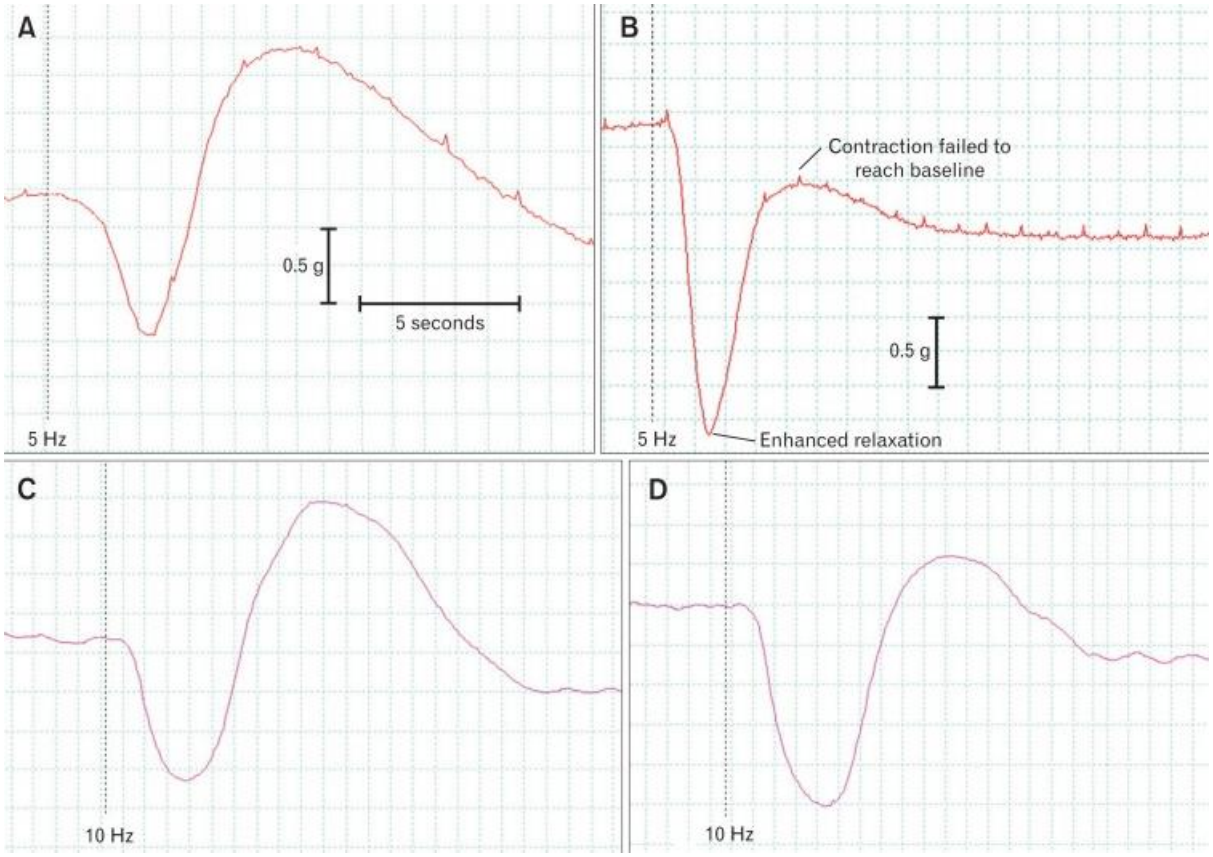

Figure 1. Experimental traces of internal anal sphincter (IAS) responses to electrical field stimulation in the absence and presence of the adrenergic neurone blocker guanethidine (10 μM; A, B) and after desensitisation of P2X receptors with α,β-mATP (10 μM; C, D). Both drugs reduced contractions and enhanced the relaxations. In the presence of guanethidine, contractions remained below the initial baseline tone.

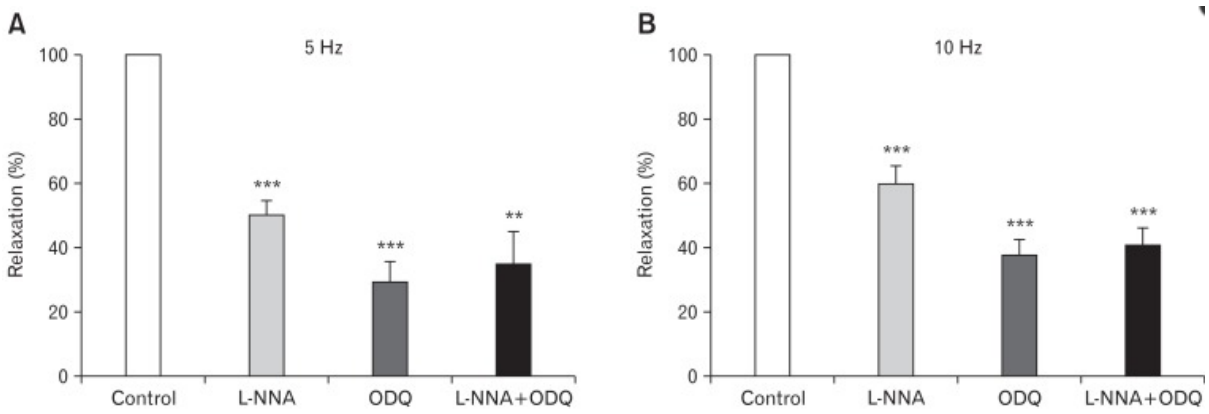

Figure 2. Effects of L-NNA (100 μM) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM), alone and in combination, on the relaxations induced by electrical field stimulation after removal of the adrenergic, cholinergic and purinergic contractile components of responses. Relaxations to electrical field stimulation (EFS) were examined at (A) 5 Hz and (B) 10 Hz. Mean responses (± SEM, n = 5-10) are expressed as a percentage of the control pre-drug response to EFS. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control values in the absence of inhibitors.

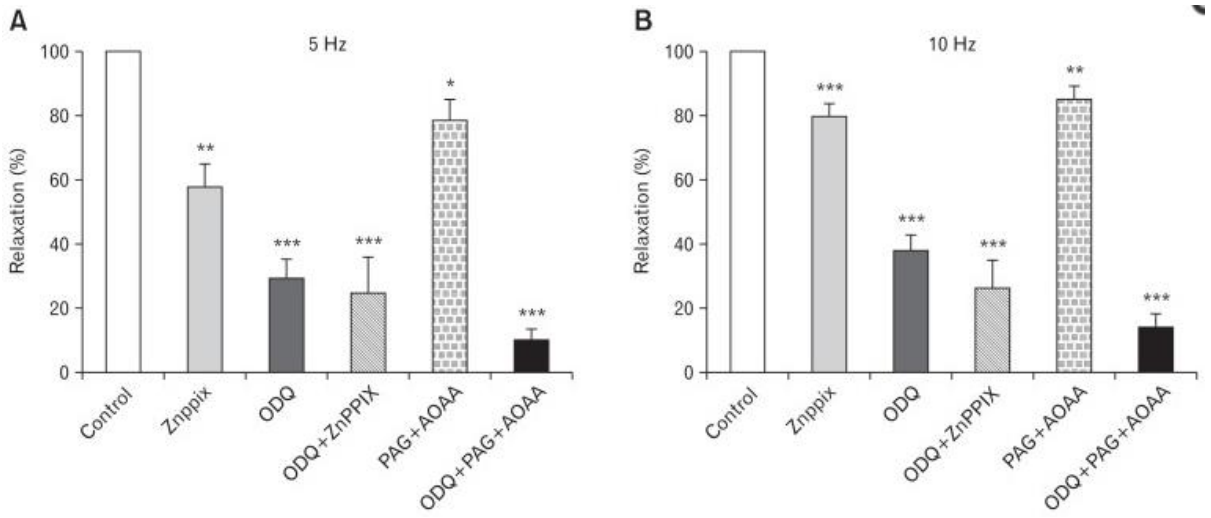

Figure 3. Effects of inhibitors of carbon monoxide (CO) and hydrogen sulfide synthesis on relaxations of the internal anal sphincter. Relaxations to electrical field stimulation (EFS) were examined after removal of the contractile components of responses with guanethidine (10 μM), atropine (1 μM), and a,β-mATP (10 μM). Responses were obtained at (A) 5 Hz and (B) 10 Hz in the absence and presence of the HO inhibitor zinc protoporphyrin IX (Znppix, 10 μM) and/or inhibitors of the hydrogen sulfide (H_2S) synthesizing enzymes, propargylglycine (PAG, 1 mM), and aminooxyacetic acid (AOAA, 30 μM). Mean responses (± SEM, n = 5-10) are expressed as a percentage of the control pre-drug response to EFS. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control values in the absence of inhibitors of CO and H_2S synthesis.

Tables

Table 1. Mean (± SEM) Contractions Developed by Yissues in Response to Electrical Field Stimulation.

Drug/frequency		Contraction (% of baseline) Absence of Drug	Contraction (% of baseline) Presence of Drug	Inhibition (% of control response)
Guanethidine (10	**5 Hz**	21.5 ± 10.3	-9.0 ± 4.8[a]	130.7 ± 47.2
μM) n = 7	**10 Hz**	55.7 ± 17.3	-8.2 ± 9.1[a]	120.0 ± 18.7
Atropine	**5 Hz**	36.1 ± 6.5	24.7 ± 7.9	NSD
(1 μM), n = 8	**10 HZ**	46.4 ± 9.2	35.3 ± 7.3	NSD
α,β-mATP (10 μM),	**5 Hz**	33.4 ± 9.6	19.2 ± 7.4[a]	44.8 ± 5.0
n = 4	**10 Hz**	42.5 ± 6.1	35.0 ± 7.8	NSD

NSD, no significant difference.

Responses are expressed as a percentage of the initial resting tone immediately prior to stimulation. In the presence of guanethidine only relaxation responses were observed (indicated by negative values). [a]$P < 0.01$ compared to responses in the absence of drug.

Table 2. Relaxation Responses Expressed As a Percentage of the Tone of the Tissue at the Time of Stimulation.

Drug/frequency		Relaxation (% of baseline)		Inhibition (% of control response)
		Absence of drug	Presence of drug	
L-NNA	**5 Hz**	56.6 ± 2.2	32.8 ± 2.8[a]	42.6 ± 4.2
(100 µM), n = 26	**10 Hz**	45.4 ± 3.7	19.9 ± 2.9[a]	60.4 ± 4.9
Indomethacin	**5 Hz**	44.9 ± 3.7	44.0 ± 3.6	1.7 ± 2.7
(5 µM), n = 14	**10 Hz**	47.1 ± 4.2	45.4 ± 4.5	4.5 ± 4.1
PheLeu-VIP	**5 Hz**	50.0 ± 4.2	48.0 ± 3.9	3.5 ± 2.5
(100 nM), n = 6	**10 Hz**	52.6 ± 4.9	52.0 ± 4.3	0.7 ± 2.2

PheLeu-VIP, [$_{D}$-p-Cl-Phe^{6},Leu^{17}]-vasoactive intestinal peptide.

An increase in inhibition indicates that the drug did not reduce the relaxation, rather it increased the relaxation. [a]$P < 0.001$ compared to response in the absence of drug.